Exhibit A

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Neuroinflammatory processes in Alzheimer's disease

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Abstract Generation of neurotoxic amyloid β peptides and their deposition along with neurofibrillary tangle formation represent key pathological hallmarks in Alzheimer's disease (AD). Recent evidence suggests that inflammation may be a third important component which, once initiated in response to neurodegeneration or dysfunction, may actively contribute to disease progression and chronicity. Various neuroinflammatory mediators including complement activators and inhibitors, chemokines, cytokines, radical oxygen species and inflammatory enzyme systems are expressed and released by microglia, astrocytes and neurons in the AD brain. Degeneration of aminergic brain stem nuclei including the locus ceruleus and the nucleus basalis of Meynert may facilitate the occurrence of inflammation in their projection areas given the antiinflammatory and neuroprotective action of their key transmitters norepinephrine and acetylcholine. While inflammation has been thought to arise secondary to degeneration, recent experiments demonstrated that inflammatory mediators may stimulate amyloid precursor protein processing by various means and therefore can establish a vicious cycle. Despite the fact that some aspects of inflammation may even be protective for bystander neurons, antiinflammatory treatment strategies should therefore be considered. Non-steroidal anti-inflammatory drugs have been shown to reduce the risk and delay the onset to develop AD. While, the precise molecular mechanism underlying this effect is still unknown, a number of possible mechanisms including cyclooxygenase 2 or γ-secretase inhibition and activation of the peroxisome proliferator activated receptor γ may alone or, more likely, in concert account for the epidemiologically observed protection.

Keywords Alzheimer's disease · Neuroinflammation · Amyloid beta · Peroxisome proliferator activated receptor gamma · Cytokines · Locus ceruleus

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Alzheimer's disease

As the most common neurodegenerative disorders, Alzheimer's disease (AD) currently affects 20–30 million individuals worldwide (Selkoe 2005). AD accounts for most cases of dementia that are diagnosed after the age of 60 years of life.

AD brains show two characteristic lesions: extracellular deposits of β-amyloid peptides, so called neuritic or senile plaques, and the intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau protein (Querfurth and LaFerla 2010).

Toxic β-amyloid peptides (Aβ) are generated by the sequential action of two proteases denoted as β-secretase (BACE1) and γ-secretase, which cleave the amyloid precursor protein (APP). Aβ exists with different carboxyl endings, from which $A\beta_{1-40}$ and $A\beta_{1-42}$ appear to be the major subtypes deposited in the brain. Aβ peptides can also be detected in normal cerebrospinal fluid and in conditioned media from various tissue culture cell lines (Shoji et al. 1992; Haass et al. 1992; Seubert et al. 1992),

suggesting that it is constantly produced and constitutively secreted. The importance of Aβ formation was revealed by dominantly inherited familial forms of AD that are linked to APP mutations in or close to the β- and γ-secretase cleavage sites (Hardy and Allsop 1991).

NFT constitute intraneuronal cytoplasmic accumulations of non-membrane-bound bundles of paired helical filaments, whose main component is the hyperphosphorylated form of the Tau protein. Tau is also found in dystrophic neurites. It is found in aggregates conjugated with ubiquitin, a property that it shares with other aggregating intraneuronal proteins, such as α-synuclein. Of importance, Aβ deposits as well as NFT can also be found in other neurodegenerative diseases and even in brains of patients without any history of cognitive or other neurological deficits (Lee et al. 2001), suggesting the contribution of additional factors to fully establish the disease.

The eventual deposition of Aβ and NFT formation may not account for all clinical symptoms of AD, particularly the most early clinical symptoms arising before neuronal degeneration is evident. Inflammatory changes are observed in AD brain overall, particularly at the amyloid deposits, which are rich in activated microglia. Once stimulated, the microglia release a wide variety of pro-inflammatory mediators including cytokines, complement components, various free radicals and nitric oxide (NO), all of which potentially contribute to further neuronal dysfunction and eventually death. These create and feed a vicious cycle that could be essential in the pathological progression of AD (Griffin et al. 1998; Griffin 2000).

Inflammation and AD

Although Aβ has been considered to play a key role in AD pathogenesis (Walsh et al. 2002b; Walsh and Selkoe 2004), it remains still uncertain whether Aβ plaques and NFT are causative for AD. These doubts are being fueled by the finding that the Aβ plaque burden only poorly correlates with the progression and severity of dementia in AD. Moreover, transgenic animals that develop widespread Aβ plaque deposition in response to mutant APP overexpression show only slight cognitive deficits (Braak and Braak 1998; Davis and Laroche 2003). Furthermore, NFT may correlate better with the decline in cognitive skills, but seem to occur as a late event and in some cases possibly downstream of Aβ accumulation. However, some experimental evidence indicates that protofibrils and oligomers of $A\beta_{1-40}$ and $A\beta_{1-42}$, rather than concrete Aβ plaques, contribute to early dendritic and synaptic injury and thereby to neuronal dysfunction (Walsh et al. 2002a).

In addition to these direct toxic effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, Aβ may promote neurodegeneration by parallel mechanisms including the activation of microglial cells and astrocytes. The induction of a microglia-driven inflammatory response results in the release of various inflammatory mediators including a whole array of neurotoxic cytokines (Tan et al. 1999; Heneka and O'Banion 2007). Once activated, microglia cells may also recruit astrocytes that actively enhance the inflammatory response to extracellular Aβ deposits. This neuroinflammatory component of AD is further characterized by a local cytokine-mediated acute-phase response, activation of the complement cascade and induction of inflammatory enzyme systems such as the inducible nitric oxide synthase (iNOS) and the prostanoid generating cyclooxygenase-2 (COX-2). Several lines of evidence suggest that all of these factors can contribute to neuronal dysfunction and cell death, either alone or in concert (Abbas et al. 2002; Bezzi et al. 2001a, b; Brown and Bal-Price 2003).

This review will discuss several aspects of neuroinflammation in AD focusing on the following questions:

1. What stimulates the inflammatory reaction in the AD brain?
2. Which cells contribute to the inflammatory component of AD and how do they interact?
3. Which pro- and antiinflammatory mediators are being released in the AD brain, and what is their supposed mechanism of action?
4. Are there any known pathogenetic factors in the AD brain that may facilitate the induction and persistence of neuroinflammatory mechanisms?
5. Is neuroinflammation just a reaction to neurodegenerative events or does it act on neurodegenerative pathomechanisms thereby establishing a vicious and self-perpetuating cycle?
6. Can antiinflammatory treatment strategies serve as a future AD therapy?

Immunostimulators in Alzheimer's disease

While minor signs of neuroinflammation can be found in the normal aging brain, the AD brain faces a much stronger activation of inflammatory systems indicating that an increasing amount or qualitatively different immunostimulants are present. Cumulative evidence suggests that Aβ peptides play a pivotal role as inducers of neuroinflammation. However, chromogranin A and several other proteins may contribute to this induction.

Amyloid β

The concept that Aβ peptide itself can induce a local inflammatory-type response received impetus from the

in vitro findings that fibrillar Aβ can bind the complement factor C1 and hence potentially activate the classical complement pathway in an antibody-independent fashion (Rogers et al. 1992). Such activated early complement factors could play an important role in the local recruitment and activation of microglial cells expressing the complement receptors CR3 and CR4 (Rozemuller et al. 1989; Eikelenboom et al. 1989). In vitro studies indicate that a certain degree of Aβ fibrillization is required for the initiation of the complement system (Snyder et al. 1994). This in vitro finding is consistent with the immunohistochemical data in AD brains showing weak or absent immunostaining for early complement components in diffuse plaques composed of non- or low-grade fibrillar Aβ peptide (Eikelenboom and Veerhuis 1996). The diffuse plaques are not associated with activated microglia and altered neurites, in contrast to the so-called classical and neuritic plaques, which are characterized by congophilic fibrillar Aβ deposits. So, the chronic inflammatory response in AD brains is seen in the plaque containing fibrillar Aβ deposits but not in the diffuse plaque with the non-congophilic low-fibrillar Aβ depositions (Rozemuller et al. 1989; Itagaki et al. 1989). For example, Aβ activates microglia by binding to the receptor for advanced glycation end products (RAGE) (Yan et al. 1998) and to other scavenger receptors (Paresce et al. 1996). Furthermore, the LPS receptor, CD14, interacts with fibrillar Aβ (Fassbender et al. 2004) and microglia kill $A\beta_{1-42}$ damaged neurons by a CD14 dependent process (Bate et al. 2004). Fibrillar Aβ has been shown to increase cytokine and nitric oxide production in microglia dependent on CD14, TLR2 and TLR4 (Jana et al. 2008; Walter et al. 2007; Reed-Geaghan et al. 2009). Another signalling pathway through which Aβ can promote inflammation has been identified by Stewart et al. (2010). These authors found that Aβ triggers inflammatory signaling through heterodimer formation of Toll-like receptor 4 and 6. Assembly of this heterodimer is regulated by binding of Aβ to scavenger receptor CD36. The involvement of CD14 and/or CD36 and Toll-like receptors in Aβ induced microglia activation strongly suggests that innate immunity is linked with AD pathology. The ability of fibrillar Aβ to trigger an inflammatory response is phenocopied in murine AD models that recapitulate Aβ plaque formation (e.g. Matsuoka et al. 2001; Jimenez et al. 2008). Paradoxically, CD14 and CD36 are among the receptors that are responsible for the clearance and phagocytosis of Aβ by microglia as well (Koenigsknecht and Landreth 2004; Liu et al. 2005). It is conceivable that phagocytosis of Aβ by microglia requires inflammatory signaling, but is obviously worn out during the course of the disease, in accordance with the finding that in 8-month-old APPswe/PS1dE9 mice expression of Aβ binding receptors and degrading enzymes in microglia is reduced (Hickman et al. 2008).

Chromogranin A

Chromogranin A (CGA) represents a secretory 48–53 kDa glycoprotein which is stored and released by neurons in brain regions relevant for several neurodegenerative diseases including AD, Parkinson's disease and amyotrophic lateral sclerosis. Of note, neuritic plaques intensely stain for CGA in AD (Rangon et al. 2003). Experiments showing that exposure of primary rat microglia to CGA resulted in rapid microglial activation characterized by profound morphological changes from an arborized to an amoeboid phenotype lead to the hypothesis that CGA may act as an important stimulator of neuroinflammation (Taupenot et al. 1996). Microglial activation was accompanied by de novo synthesis of iNOS and subsequent production of NO (Taupenot et al. 1996). Importantly, CGA was equally or more effective in stimulating iNOS derived NO release relative to microglial stimulation with bacterial lipopolysaccharide. Activation of microglial cells with CGA caused neuronal cell death, however, a direct link between NO or tumor necrosis factor α (TNFα) release and neurodegeneration has not been found in this model (Ciesielski-Treska et al. 1998a, b).

Cellular components of neuroinflammation in Alzheimer's disease

Microglia cells represent the brain innate immune system and hence the first line of defense when challenged by bacterial, viral or fungal infection. Although these functions are of major importance and beneficial, it has become clear that microglial activation may also be evoked by endogenous proteins and can significantly contribute to neuronal damage. Along with microglia, astrocytes and even neurons are directly reacting and contributing to the chronic neuroinflammatory changes in AD.

Microglia

Microglia cells constitute around 10% of all cells in the nervous system. They represent the innate immune system in the brain and thus the first line of defense against invading pathogens and serve as specialized sensors for brain tissue injury (Ransohoff 2009; Hanisch and Kettenmann 2007). Under pathological situations, such as neurodegenerative disease, stroke, traumatic injury and tumor invasion, these cells become activated, migrate to and surround damaged or dead cells, and subsequently clear cellular debris from the area, similar to the phagocytic

active macrophages of the peripheral immune system. During the course of neurodegeneration, not only the morphological phenotype of microglia is changing but also their overall number. To date it remains unclear whether the increase in microglia arises from local self renewal as suggested for the SOD1 transgenic mouse model of ALS and the facial axotomy model (Ajami et al. 2007) or is caused by the invasion of peripheral monocytes in response to central inflammation as shown in an AD transgenic mouse model (Simard and Rivest 2007).

Activated microglia up-regulate a variety of surface receptors, including the major histocompatibility complex and complement receptors (Liu and Hong 2003). They also undergo dramatic morphological changes from a ramified phenotype to motile activated amoeboid cells (Kreutzberg 1996). Once immunostimulated in response to neurodegenerative events, these microglia cells release a variety of proinflammatory mediators including cytokines, reactive oxygen species, complement factors, neurotoxic secretory products, free radical species and NO, all of which can contribute to neuronal dysfunction and cell death, ultimately creating a vicious cycle.

Next to the classical neuropathological features of AD, namely Aβ deposition and NFT formation, neuroinflammatory changes have been identified as the third important component of the disease. The inflammatory reactions of microglia and astrocytes are intimately associated with the pathogenesis and progress of AD. The activated microglia is associated with neuritic plaques (McGeer and McGeer 1999) and secretes a wide variety of pro-inflammatory molecules (Heneka and O'Banion 2007). Furthermore activated microglia is implicated in active phagocytosis of Aβ, thus counterbalancing the Aβ load (Frautschy et al. 1998; Bolmont et al. 2008). The latter finding, however, has been questioned by a recent study using an microglial ablation system through herpes simplex virus thymidine kinase expression under the control of the CD11b promoter and ganciclovir treatment in vivo (Grathwohl et al. 2009). In this work, ablation of microglia in an AD mouse model, that develops plaque pathology already at 2–3 month of age, did not affect plaque load. One interpretation of this finding could be that the occurrence of toxic amyloid species along with inflammatory mediators was leading to an early loss of microglial key functions including phagocytosis, which results in a paralysis of these cells. Ablation of these paralyzed microglia, of course would not result in any change of Aβ plaque load since these cells have by far earlier stopped to limit plaque build up in such a scenario.

Nevertheless, there are no doubts that the activation of microglia occurs in response to formation of amyloid plaques. Several amyloid peptides and APP itself can act as potent glial activators (Barger and Harmon 1997; Dickson et al. 1993; Schubert et al. 2000), and disruption of the APP gene and its proteolytic products delay and decrease microglial activation (DeGiorgio et al. 2002). Microglial cells have been suggested to be preferentially associated with certain amyloid plaque types indicating that plaque development and the degree of microglial reaction are interrelated (D'Andrea et al. 2004). However, it remains unclear whether Aβ plaque deposition is an absolute requirement for microglial activation, or whether this can already be evoked by soluble and toxic Aβ species. This hypothesis finds support by a recent study where focal activation of microglial cells becomes apparent at 3 month of age in APP V717I transgenic mice, which usually start to deposit Aβ in plaque-like structures much later around 10–12 months (Heneka et al. 2005a). In contrast, studies using in vivo multiphoton microscopy using 5–6 month olf B6C3-YFP transgenic mice (bearing APPswe and PS1d9x-YFP genes) suggested that microglia are recruited to Aβ plaques only after they have been formed (Meyer-Luehmann et al. 2008).

The mechanisms of microglial activation by Aβ depositions have not been fully elucidated yet, although several receptor systems are directly implicated in this process (Fig. 1). In particular, activation of microglia requires P2X7 purinoceptors and Ca2+ signaling. Exposure of cultured microglial cells to Aβ_{25-35} triggers Ca2+ influx, IL-1β release and P2X7-dependent membrane permeabilisation, which was absent in cells prepared from P2X7 KO

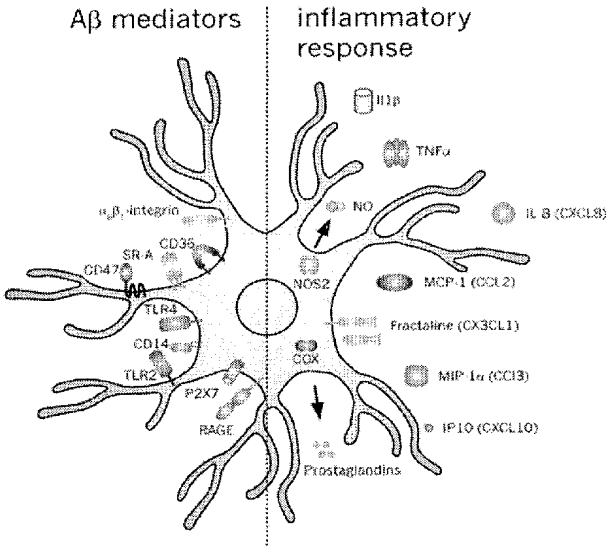

Fig. 1 Pathways of microglial activation by Aβ. Aβ is able to stimulate microglia by a variety of cell surface molecules (*left*), resulting in the secretion of inflammatory molecules (*right*). These molecules include members of the cytokine family, others are small compounds of the COX metabolism (prostaglandins) or short-lived molecules like nitric oxide

mice (Sanz et al. 2009). Furthermore, intra-hippocampal injection of $A\beta_{1-42}$ failed to induce microglial activation (as judged by IL-1β accumulation) in animals deficient for P2X7 receptors (Sanz et al. 2009).

Activation of microglial cells by aggregated Aβ also involves Toll-like receptors (Udan et al. 2008; Okun et al. 2009) of the TLR2 and TLR4 type. Expression of TLR2 and TLR4 receptors were upregulated in both AD brains and in related transgenic mouse models of AD and plaque associated microglia have increased levels of mRNA coding for TLR2, -4, -5, -7 and -9 (Frank et al. 2009). A spontaneous loss-of-function mutation in the TLR4 gene significantly reduced Aβ-induced microglial activation (Walter et al. 2007). Exposure of microglial cultures to Aβ also stimulated TLR2 receptors, while inhibiting TLR9 receptors (Lotz et al. 2005). Stimulation of TLR-associated signaling systems may have dual effects in AD progression. On one hand, activation of TLRs increases microglial phagocytosis of Aβ (this involves p38 MAPK signaling and expression of G-protein-coupled formyl peptide receptor-like 2, mFPR2; the latter likely being the sensor for Aβ (Chen et al. 2006; Iribarren et al. 2005). Loss of function mutation of TLR4 or knockout of TLR2 have been found to increase plaque load in murine AD models. Increased Aβ deposition in APP/PS1 mice knockout for TLR2 was accompanied by impaired memory performance (Richard et al. 2008). Stimulation of Toll-like receptor 9 signaling with oligodeoxynucleotides has been shown to decrease plaque load and improve radial maze performance in Tg2576 mice (Scholtzova et al. 2009). It is interesting to note that activation of TLR9 triggered microglial clearance of oligomeric Aβ and attenuated neurotoxicity in neuron-microglia co-cultures exposed to oligomeric Aβ (Doi et al. 2009). At the same time, however, overstimulation of TLRs may trigger excessive release of cytokines, proteases and other cytotoxins thus promoting neural cell death (Okun et al. 2009). Aβ stimulates a nuclear factor kappa B (NFκB)-dependent pathway that is required for cytokine gene transcription (Combs et al. 2001) within activated microglia and reactive astrocytes. Not only Aβ, but also the carboxy-terminal 100 amino acids of APP (CT100), which is also present in senile plaques, can induce astrocytosis and neuronal death. CT100 exposure results in activation of the mitogen-activated protein kinase (MAPK) pathways as well as NFκB (Bach et al. 2001). Additionally, other proteins involved in APP processing have been implicated in the inflammatory response. Loss of presenilin function in presenilin conditional knockout mice leads to differential up-regulation of inflammatory markers in the cerebral cortex, such as strong microglial activation, complement component C1q, and cathepsin S (Beglopoulos et al. 2004).

Once stimulated, microglial participate in the generation and release of a wide range of inflammatory mediators including complement factors, chemokines and cytokines. The complement system represents a complex and tightly regulated attack cascade designed to destroy invaders and assist in the phagocytosis of waste, one of the key microglial tasks under physiological and pathophysiological conditions. The components of this system carry out four major functions: recognition, opsonization, inflammatory stimulation and direct killing through the membrane attack complex (MAC) (McGeer and McGeer 2002). In addition to triggering the generation of a membranolytic complex, complement proteins interact with cell surface receptors to promote a local inflammatory response that contributes to the protection and healing of the host. Microglial complement activation causes inflammation and cell damage, yet it is essential for eliminating cell debris and potentially toxic protein aggregates. The complement system consists of some 30 fluid-phase and cell-membrane associated proteins that can be activated by three different routes: the classical pathway (involving C1q, C1r, C1s, C4, C2, and C3 components) is activated primarily by the interaction of C1q with immune complexes (antibody–antigen), but activation can also be achieved after interaction of C1q with non-immune molecules such as DNA, RNA, C-reactive protein, serum amyloid P, bacterial lipopolysaccharides, some fungal and virus membranes, and most importantly, fibrillar Aβ. The initiation of the alternative pathway (involving C3, factor B, factor D, and properdin) does not require the presence of immune complexes and leads to the deposition of C3 fragments on target cells. Mannose-binding lectin (MBL), a lectin homologous to C1q, can recognize carbohydrates such as mannose and N-acetylglucosamine on pathogens and initiate the complement pathway independently of both the classical and the alternative activation pathways. Like the C1 complex in the classical pathway, MBL is associated with two serine proteases that cleave C4 and C2 components, leading to the formation of the classical C3 convertase (van Beek et al. 2003).

Microglial cells can produce complement proteins to recognize and kill pathogens locally. Studies using quantitative PCR have shown locally upregulated complement mRNA in AD brain, especially in the areas of primary pathology: entorhinal cortex, hippocampus, and midtemporal gyrus (Yasojima et al. 1999b). Numerous groups have reported the association of complement proteins of the classical pathway, particularly the MAC, with amyloid plaques and NFT in AD brains (Webster et al. 1997b). Information about the functional role comes from studies of mutant mice lacking complement proteins, which suggest that impaired phagocytosis can result in immune-mediated tissue damage and inflammation (Botto 1998; Taylor et al. 2000). However, the complement system may be Janus faced and also provide beneficial action to the

brain during AD, Thus, Wyss-Coray et al. (2002) demonstrated that complement activation can protect against Aβ-induced toxicity and may reduce the accumulation or promote the clearance of senile plaques. AD mice expressing a soluble form of the complement inhibitor Crry, which blocks C3 activation, under the control of the glial fibrillary acidic protein promoter displayed higher Aβ deposition and more prominent neurodegeneration than age-matched control mice. However, more recently it was reported that transgenic mouse models of AD lacking C1q showed reduced pathology, consisting of decreased numbers of activated microglia and improved neuronal integrity, without changes in plaque. These data suggest that at stages when fibrillar plaque pathology is present, C1q exerts a detrimental effect on neuronal integrity, most likely through the activation of the classical complement cascade.

The finding that blocking C3 activation increases plaque load indicates that microglia at least to some extent has the ability to clear Aβ. Why plaque load did not alter but plaques recruited less microglia in the C1q deficient mice, is puzzling, but may point to a switch to an alternative activation state. A recent publication demonstrated that a C5a antagonist reduced plaque load and microglial activation in 3xTg mice (Fonseca et al. 2009). Since C5a is a late inflammatory marker, it may be postulated that for the clearance of Aβ some activation of microglia is necessary but that sustained strong activation leads to a neurotoxic phenotype. Targetting C5a in AD is attractive since it would leave upstream possibly beneficial complement components unaffected. It has also been shown that complement factor H expression is downregulated in AD patients via a NFkB sensitive micro RNA mediated regulation (Lukiw et al. 2008), which may result in increased complement activity on healthy host cells.

In AD, unlike the aforementioned neurological disorders characterized by leukocyte infiltration, abnormal or excessive migration of inflammatory cells into the CNS has not been definitively shown to occur. Nonetheless, there is growing evidence that chemokines and chemokine receptors are upregulated in resident CNS cells in AD brain (Ransohoff 2009), and chemokines may contribute to plaque-associated inflammation and neurodegeneration. Upregulation of CXCR2 expression has been observed on some dystrophic neurites in senile plaques (Xia and Hyman 1999; Horuk et al. 1997). In addition, the expression of CCR3 and CCR5 is increased on some reactive microglia in AD, and MIP-1α is found in a subpopulation of reactive astrocytes (Xia et al. 1998). MCP-1 has also been localized to mature senile plaques and reactive microglia, but is not found in immature senile plaques. Furthermore, in vitro studies have demonstrated the ability of Aβ to stimulate the production of IL-8, MCP-1, MIP-1α and MIP-1β from human monocytes (Meda et al. 1999). For example, microglia cultured from rapid autopsies of AD and ND patients exhibit significant, dose-dependent increases in IL-8, MCP-1 and MIP-1 α after exposure to Aβ (Lue et al. 2001). Although more studies are certainly needed, it is likely that plaque-associated chemokine production plays a role in the recruitment and accumulation of microglia to A β plaques. Future studies using targeted disruption of chemokines and chemokine receptors in mouse models of AD should help clarify the role of chemokines in plaque-associated inflammation and neurodegeneration.

Microglia derived cytokines associated with AD include several interleukins (ILs), TNF-α and TGFβ amongst others. In general, cytokine production is increased in inflammatory states and they function by regulating the intensity and duration of the immune response (Tuppo and Arias 2005; Heneka and O'Banion 2007). Thus, IL-1 induces IL-6 production, stimulates iNOS activity (Rossi and Bianchini 1996), and induces the production of M-CSF (Frei et al. 1992; Aloisi et al. 1992; Thery et al. 1992). In addition, IL-1 enhances neuronal acetylcholinesterase activity, microglial activation and additional glial IL-1 production, with consequent activation, and expression of the cytokine S100β by astrocytes, thereby establishing a self propagating cycle (Griffin 2000; Mrak and Griffin 2001). IL-6 promotes astrogliosis (Selmaj et al. 1990), activates microglia (Heyser et al. 1997), and stimulates the production of acute phase proteins, like C-reactive protein and complement components (Castell et al. 1989). IL-6 knockout mice exhibit a facilitation of radial maze learning over 30 days and show a faster acquisition, suggesting a possible negative regulation of memory formation and consolidation processes by IL-6 (Braida et al. 2004). TNF-α has both pro-apoptotic and anti-apoptotic effects. This proinflammatory cytokine accounts for most of the neurotoxic activity secreted by monocytes and microglia (Combs et al. 2001). On the other hand, TNF-α has been reported to have neuroprotective properties in the AD brain.

In addition to the general role of cytokines, AD-specific interactions of certain cytokines with the APP processing pathway and Aβ may be pathophysiologically relevant. For example, IL-1 can regulate APP processing and Aβ production in vitro (Blasko et al. 1999). In turn, fibrillar Aβ has been reported to increase neurotoxic secretory products, proinflammatory cytokines and reactive oxygen species (Eikelenboom and van Gool 2004; Eikelenboom et al. 1994; McGeer and McGeer 1995). Cultured rat cortical glia exhibit elevated IL-6 mRNA after exposure to the carboxy-terminal 105 amino acids of APP (Chong 1997). IL-1, IL-6, TNF-α MIP-1α and MCP-1 increase in a dose-dependent manner after cultured microglia are incubated with Aβ (Floden and Combs 2006; Lindberg et al. 2005; Benveniste et al. 2001; Butovsky et al. 2005; Veerhuis et al. 2005;

Hanisch 2002). Together, Aβ stimulated production of interleukins and other cytokines and chemokines and their feedback activation of APP production or BACE1 (Sastre et al. 2003, 2006) may establish a self perpetuating, vicious cycle. A second general category of cytokine action is manifested by inhibitory, anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1Ra), IL-4, IL-10 and TGF- β. Some of these are reportedly elevated in AD, consistent with induction of homeostatic mechanisms in neuroinflammation (Grammas and Ovase 2001; Szczepanik et al. 2001b; Rota et al. 2006). The use of anti-inflammatory cytokines such as IL-4 and TGF-β could be beneficial, because they are able to inhibit CD40 and class II MHC by restricting their expression and activity (Benveniste et al. 2001). Another potentially beneficial effect of IL-4 that counteracts AD pathology has recently been reveiled (Shimizu et al. 2008). They observed that IL-4 selectively induces the clearance of oligomeric Aβ by rat type 2 microglia (Shimizu et al. 2008), which was dependent on the expression of CD36. However, overexpression of TGFβ in transgenic mice leads to changes in the microvasculature, including age related amyloid deposition (Wyss-Coray et al. 2000), reflecting the multi-functional nature of many cytokines. In addition to the above described evidence from the analysis of human brain tissue, cell culture and transgenic animal studies, an association of AD with several polymorphisms of proinflammatory genes has been described, including IL-1 (Nicoll et al. 2000), IL-6 (Papassotiropoulos et al. 1999), TNF-α (McCusker et al. 2001; Perry et al. 2001b), and α1-antichymotrypsin, an acute phase protein (Kamboh et al. 1995). However, none of the various members of the interleukin cytokine family that are associated with AD actually map to chromosomal regions with evidence of genetic linkage (Tanzi and Bertram 2005). Thus, although inflammation and the upregulation of inflammatory mediators like the interleukins are regularly observed in AD brain, it appears less likely that variation at the genomic level of these proteins makes a large contribution to AD risk in general. Whereas overall inflammatory responses in AD may be detrimental, a few recent publications have shown that overexpression of certain proinflammatory cytokines (IL-1β, IL-6) activate microglia and reduce plaque deposition in animal models of AD (Shaftel et al. 2007; Chakrabarty et al. 2010). This is in accordance with other experimental manipulations that enhance inflammatory responses and reduce Aβ load. It is conceivable that the extent in which microglia is able to clear Aβ during the progress of AD is too limited and that boosting this ability can prevent plaque formation. However, such manipulations in themselves can create a neurotoxic environment in the brain. For instance, it has been shown that the IL-1β overexpressing mice are behaviorally impaired (Hein et al. 2010; Moore et al. 2009) and TNF-α overexpression in an animal model of AD (3xTg mice) has been shown to cause neuronal loss, but plaque load was not assessed in the latter study (Janelsins et al. 2008). Results of experiments with overexpression or knockout of cytokines or cytokine receptors have to be taken with care, moreover, since cytokine or their receptors can be found on non-inflammatory brain cells and may execute non-inflammatory functions as well. For example, knockout of the TNF death receptor in APP23 mice leads to reduced expression of BACE1, reduced Aβ production and deposition in APP23 mice (He et al. 2007). Therefore, manipulations of the inflammatory cascades should be always checked not to affect APP processing itself before any firm conclusions about an independent role of the respective inflammatory marker can be drawn. Next to complement factors, chemokines and cytokines, activated microglia can also serve as a chief source of prostanoids. Two isoforms of cyclooxygenases, the mainly constitutively expressed COX-1 and the inducible COX-2, catalyze key steps of prostanoid synthesis in mammalian cells. Downstream of both COX-1 and COX-2, several other enzymes regulate the generation of a whole spectrum of prostanoids, some of which may be neuroprotective and others neurodestructive. Thus, the composition and proportion of all prostanoids together may actually determine whether the activity of COX enzymes is beneficial or detrimental.

In vitro, LPS activated microglial cells and IL-1β-stimulated astroglial cells are capable of synthesizing COX-2 (Bauer et al. 1997; O'Banion et al. 1996; Almer et al. 2001). In contrast to peripheral monocytes, cultured rat microglia cells do not synthesize COX-2 in response to IL-1β or IL-6 (Bauer et al. 1997), suggesting that COX-2 regulation differs between CNS and peripheral cells. In rat microglial cell cultures, the major enzymatic product of COX-2 appears to be prostaglandin E2 (PGE2). Because PGE2 itself is able to induce COX-2 in microglial cells (Minghetti et al. 1997), some sort of autocrine or paracrine amplification of the COX-2 induction in microglial cells or a spreading of COX-2 expression between neurons and microglial cells seems possible. PGE2 acts on four different receptors: EP1–EP4 (Narumiya et al. 1999). EP1 and EP2 receptors have been detected in cultured microglia, while EP3 receptors are also present in activated microglia in vivo (Slawik et al. 2004). Microglial EP2 receptors inhibit phagocytosis and enhance neurotoxic activities of microglia (Shie et al. 2005a, b). PGE2 may also act on the neuronal EP2 receptor, which is involved in apoptosis, although investigations of the role of neuronal EP2 activation on neuronal cell death have yielded conflicting results and suggest a neuroprotective role of neuronal EP2 stimulation under several pathophysiological circumstances (Bilak et al. 2004; Lee et al. 2004; McCullough et al. 2004; Takadera et al. 2004). This is further

exemplified in a recent report where knockout of EP2 in a double transgenic (APP/PS1) mouse led to decreased evidence of oxidative stress and descreased Aβ production, associated with lower levels of BACE (Liang et al. 2005). In conclusion, neuronal and glial secretion of PGE2 may impair phagocytotic clearance of Aβ by binding to the microglia EP2 receptor and enhancing microglial toxicity. However, the role of PGE2 in neurodegeneration may be by far more complex due to the presence of other EP receptor subtypes on microglial cells and the effects of PGE2 on other cell types. Neuronal death elicited by excitotoxins is elevated in transgenic animals with high expression of COX-2, suggesting that COX-2 expression may further interact with other pathogenic mechanisms (Kelley et al. 1999).

It should be noted that some aspects of microglia function may be beneficial, since activated microglia are able to reduce Aβ accumulation by increasing its phagocytosis, clearance and degradation (Frautschy et al. 1998; Qiu et al. 1998). Moreover, secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides are constitutively degraded by the insulin degrading enzyme (IDE), a metalloproteinase released by microglia and neural cells. Finally, microglia can also secrete several neurotrophic factors, such as the glia-derived neurotrophic factor (GDNF), which exert a well documented neuroprotective function (Liu and Hong 2003).

Finally, it has been shown that bone marrow-derived cells are able to cross the blood–brain barrier (BBB) and to differentiate into fully functional microglia afterwards (Malm et al. 2005; Simard and Rivest 2004; Hess et al. 2004). In this context it has been demonstrated that engrafted monocytes are able to infiltrate the brain and to restrict the formation of amyloid plaques (Simard et al. 2006). One has to mention that there are concerns, whether this represents an physiological event or that such a phenomenon results from the damage of the BBB during the generation of chimeric mice using either BMSC transplantation or irradiation (Mildner et al. 2007; Ajami et al. 2007).

Astrocytes

The glial involvement in the pathogenesis of AD was initially suggested by Alois Alzheimer himself. He had demonstrated that the neuritic plaques (extracellular deposits of fibrillar Aβ) together with tau NFT represent the major histopathological markers of AD. In addition, AD brains are characterized by prominent astrogliosis, mostly observed in the cells surrounding amyloid plaques with processes of activated astrocytes participating in formation of neuritic plaques (Nagele et al. 2003; Rodriguez et al. 2009).

The Aβ peptide represent an activating signal for astrocytes; exposure of cultured glial cells to aggregated Aβ or to amyloid plaques isolated from human AD brains trigger reactive astrogliosis (Dewitt et al. 1998). Aβ also induces functional changes in astrocytes in vitro: $A\beta_{1-42}$ and its toxic fragment $A\beta_{25-35}$ induced spontaneous $[Ca2+]_i$ elevations and $[Ca2+]_i$ oscillations in astrocytes growing in mixed astroglial-neuronal cultures. The Aβ-induced $[Ca2+]_i$ oscillations lasted for many hours and were linked to neuronal death, which occurred 24 h after administration of Aβ to the cultures. Inhibition of $[Ca2+]_i$ oscillations prevented neuronal death (Abramov et al. 2003). In the same mixed culture model Aβ was also shown to induce mitochondrial depolarisation and oxidative stress in astrocytes; the release of reactive oxygen species from stressed astrocytes caused neuronal death. Recently, (Allaman et al. 2010) reported that aggregated $A\beta_{1-42}$ was taken up by astrocytes and caused metabolic disturbances and production of hydrogen peroxide. Astrocytes pretreated with Aβ were toxic to neurons in co-cultures. Metabolic disturbances as well as toxicity could be prevented by a PI-3 kinase inhibitor.

The abnormalities in astroglial Ca2+ signaling were also observed in the brains of transgenic AD mice. In these experiments, employing in vivo multiphoton-confocal-microscopy, the general elevation of resting $[Ca2+]_i$ was observed throughout the astroglial syncytia. In addition, astrocytes located in the vicinity of plaques triggered spontaneous long-distance propagating Ca2+ waves, which were absent in control animals (Kuchibhotla et al. 2009).

Participation of astrocytes in plaques formation initiated the hypothesis of an Aβ-clearing role of astroglia (Nagele et al. 2003; Wyss-Coray et al. 2003) with subsequent astroglial degeneration triggered by accumulated Aβ. Indeed plating of isolated healthy astrocytes on the slices prepared from transgenic (APP) AD mice resulted in migration of astrocytes towards the plaques with subsequent accumulation and degradation of Aβ. In support of this finding, recent evidence suggests that astroglial cells are able to phagocytose Aβ peptides, a process which may depend on their apolipoprotein E (ApoE) status, suggesting that ApoE polymorphisms may influence the risk to develop AD by affecting astroglial Aβ phagocytosis. In contrast, however, endogenous astrocytes surrounding the Aβ plaques were unable to accumulate and remove Aβ (Wyss-Coray et al. 2003).

In the triple transgenic mouse model of AD [3xTg-AD; harboring the mutant genes for amyloid precursor protein (APPSwe), presenilin 1PS1M146V and tauP301L (Oddo et al. 2003)] very little, if any Aβ accumulation by reactive astrocytes was observed (Rodriguez et al. 2009). These

data clearly indicate the phenotypic difference between normal astroglia and astrocytes affected by AD pathology. Another kind of phenotypic difference was observed in astrocytes from AD model expressing double mutated K670N-M671L APP; these astrocytes expressed γ-secretase becoming thus possible producers of Aβ (Hartlage-Rubsamen et al. 2003) in line with findings by others (Rossner et al. 2005; Heneka et al. 2005a). While it remains unclear to which degree astrocyte activation contributes to Aβ generation or its clearance, it seems apparent that astrocytes contribute to the inflammatory component of AD. For example, astrocytes have been shown to express iNOS and the L-arginine-supplying enzyme argininosuccinate synthetase and consequently contribute to NO- and peroxynitrite mediated neurotoxicity (Heneka et al. 2001). Although astrocytes serve as a constant and important source of neurotrophic factors under physiological conditions, in vitro and in vivo experiments suggest that chronically activated inflammatory astrocytes may not generate significant amounts of these molecules (Nagatsu and Sawada 2005).

Reactive and pathologically changed astrocytes are also responsible for failures in the functional activity of neuronal-glial-vascular units. Indeed, the vascular dysfunctions, perivascular amyloidosis and compromised blood–brain barrier are inseparable parts of AD pathology (Bell and Zlokovic 2009). How astroglial cells are participating in these changes remains, however, and open question.

The astrogliosis however is not the only astroglial reaction in the AD brains. In a recent study performed on different regions of the brains of triple-transgenic [3x-Tg-AD—(Oddo et al. 2003)] mice, both astrogliosis and astroglial atrophy were found [(Rodriguez et al. 2009); Rodriguez and Verkhratsky paper in preparation]. The decrease in complexity of astrocytes, which indicated their atrophy, begun to be observed before the formation and consolidation of neuritic plaques. In plaque infested brains the reactive astrocytes were concentrated around the Aβ plaques, whereas astroglial cells distant to the plaques had an atrophic features.

Neurons

While neurons were traditionally believed to be passive bystanders in neuroinflammation, more recent evidence suggests that neurons themselves are capable of producing inflammatory mediators. Thus, neurons can serve as source of complement, COX-2-derived prostanoids (Yermakova et al. 1999), and several cytokines including IL-1β, IL-6, and TNF-α (Botchkina et al. 1997; Breder et al. 1993; Gong et al. 1998; Murphy et al. 1999; Orzylowska et al. 1999; Suzuki et al. 1999; Tchelingerian et al. 1994; Yan et al. 1995; Hoozemans et al. 2004; Aloisi et al. 1992) and M-CSF (Du et al. 1997). Although COX-2 expression is driven by physiological synaptic activity (Yamagata et al. 1993; Yermakova and O'Banion 2000), it appears possible that neurons themselves may exacerbate local inflammatory reactions and thus contribute to their own destruction in AD. As a further factor, expression of the inflammatory induced enzyme iNOS has been described in degenerating neurons in AD brains (Vodovotz et al. 1996; Lee et al. 1999; Heneka et al. 2001), and compelling evidence exists for iNOS related long-term NO release and NO dependent peroxynitrite formation during the course of AD (Smith et al. 1997). Glial and neuronal derived NO and peroxynitrite have been demonstrated to cause neuronal dysfunction and cell death in vitro and in vivo (Boje and Arora 1992; Heneka et al. 1998). Alternatively, some of the classical pro-inflammatory mediators such as TNF-α and low concentrations of NO may actually confer neuroprotection rather than destruction in the brain and therefore constitute a defense mechanism against local inflammatory reactions.

Finally, it is reported that prostaglandin E2 is able to increase Aβ production in neuronal cells mediated by internalization of the EP4 receptor (Hoshino et al. 2009).

Pro- and antiinflammatory mediators

The neuroinflammatory response observed in AD is characterized by a whole array of pro- and antiinflammatory mediators including members of the complement cascade, chemo- and cytokines as well as inflammatory enzyme systems. Several of these factors may promote neurodegenerative mechanisms while others may rather limit ongoing inflammatory changes or even exert beneficial neurotrophic effects. Thus, not a single mediator but rather the entire spectrum of inflammatory agents will determine whether beneficial or detrimental effects prevail.

Complement

The complement system represents a complex and tightly regulated attack cascade designed to destroy invaders and assist in the phagocytosis of waste materials. The components of this system carry out four major functions: recognition, opsonization, inflammatory stimulation and direct killing through the membrane attack complex (MAC) (McGeer and McGeer 2002). In addition to triggering the generation of a membranolytic complex, complement proteins interact with cell surface receptors to promote a local inflammatory response that contributes to the protection and healing of the host. Complement activation causes inflammation and cell damage, yet it is essential for eliminating cell debris and potentially toxic protein

aggregates (Shen and Meri 2003). Furthermore, the complement system may contribute to synapse remodeling through marking weak synapses for removal presumably by microglia (Stevens et al. 2007).

The complement system consists of some 30 fluid-phase and cell-membrane associated proteins that can be activated by three different routes: The classical pathway (involving C1q, C1r, C1s, C4, C2, and C3 components) is activated primarily by the interaction of C1q with immune complexes (antibody-antigen), but activation can also be achieved after interaction of C1q with non-immune molecules such as DNA, RNA, C-reactive protein, serum amyloid P, bacterial lipopolysaccharides, some fungal and virus membranes, and as already mentioned, fibrillar Aβ. The initiation of the alternative pathway (involving C3, factor B, factor D, and properdin) does not require the presence of immune complexes and leads to the deposition of C3 fragments on target cells. Mannose-binding lectin (MBL), a lectin homologous to C1q, can recognize carbohydrates such as mannose and *N*-acetylglucosamine on pathogens and initiate the complement pathway independently of both the classical and the alternative activation pathways. Like the C1 complex in the classical pathway, MBL is associated with two serine proteases that cleave C4 and C2 components, leading to the formation of the classical C3 convertase (van Beek et al. 2003).

Various brain cells can produce complement proteins to recognize and kill pathogens locally. Cell lines and primary cultures of human origin were used to show that glial and neuronal cells could produce most complement proteins, particularly after stimulation with inflammatory cytokines (Gasque et al. 1995). Studies using RT-PCR have shown locally upregulated complement mRNA in AD brain, especially in the areas of primary pathology: entorhinal cortex, hippocampus, and midtemporal gyrus (Yasojima et al. 1999b). Numerous groups have reported the association of complement proteins of the classical pathway, particularly the MAC, with amyloid plaques and NFT in AD brains (Webster et al. 1997a).

Studies of mutant mice lacking complement proteins suggest that impaired phagocytosis can result in immune-mediated tissue damage and inflammation (Botto 1998; Taylor et al. 2000). Wyss-Coray et al. (2002) demonstrated that complement activation can protect against Aβ-induced toxicity and may reduce the accumulation or promote the clearance of senile plaques. AD mice expressing a soluble form of the complement inhibitor Crry, which blocks C3 activation, under the control of the glial fibrillary acidic protein promoter displayed higher Aβ deposition and more prominent neurodegeneration than age-matched control mice.

However, more recently it was reported that transgenic mouse models of AD lacking C1q showed reduced pathology, consisting of decreased numbers of activated glia and improved neuronal integrity, without changes in plaque area. These data suggest that at stages when fibrillar plaque pathology is present, C1q exerts a detrimental effect on neuronal integrity, most likely through the activation of the classical complement cascade and the enhancement of inflammation (Fonseca et al. 2004). In line with this, one could hypothesize that under neurodegenerative conditions, otherwise physiological neurodevelopmental systems such as described by Stevens and colleagues (see above) may be reactivated.

Chemokines

Recent experiments have focused on understanding the role of chemokines and their receptors in AD neuroinflammation. The chemokine family consists of over 50 different molecules that confer chemotaxis, tissue extravasation, and functional modulation of leukocyte function during inflammation (Luster 1998; Owens et al. 2005). The importance of chemokine generation in AD brain is underscored by the fact that these molecules may potently regulate microglial migration and recruitment of astrocytes to the area of neuroinflammation, and thus are responsible for the extent of local inflammation. In addition, recent studies using chimeric mice grafted with green fluorescent protein expressing bone marrow cells, indicate that many of the so called "microglia" represent invading macrophages from peripheral blood (Stalder et al. 2005; Simard et al. 2006) suggesting a chemotactic stimulus from the brain. Whether this represents a technical artefact due to radiation induced alterations at the brain barrier or in fact occurs in human AD as part of the pathogenetic sequale is yet to be determined. The CXC subclass of chemokines is considered one of the two major chemokine subfamilies and its members (e.g. IL-8) are primarily chemotactic for neutrophils and endothelial cells. The conserved glutamate–leucine–arginine (ELR) motif within the receptor-binding domain of these proteins distinguishes them from non-ELR CXC chemokines such as IP-10, which primarily attract activated T cells (Strieter et al. 1995). The CC chemokine subfamily, whose members include MIP-1α, MCP-1, and RANTES, do not affect neutrophils but are chemotactic for monocytes/macrophages, T lymphocytes, basophils and eosinophils. Seven transmembrane, G-protein-coupled cell-surface receptors mediate the biological activities of chemokines and these receptors are named according to their chemokine subfamily classification. At present there are five known CXC receptors (CXCR1 to CXCR5) and nine CC receptors (CCR1 to CCR9) (Charo and Ransohoff 2006).

While it has been reported that chemokines exert physiological action in healthy brain (Hesselgesser and

Horuk 1999), the majority of studies have focused on the expression pattern of chemokines and their respective receptors in neurological diseases such as multiple sclerosis, traumatic brain injury and stroke. All of these disorders share disruption of the blood brain barrier as an important pathogenetic event subsequently allowing peripheral leukocytes to infiltrate the lesion site (Glabinski and Ransohoff 1999). In contrast, no convincing evidence exists for blood brain barrier disruption or significant leukocyte infiltration in the AD brain. However, several chemokines and chemokine receptors have been found to be upregulated in the AD brain (Xia and Hyman 1999), and chemokines may play an important role for recruiting microglia and astroglia to sites of Aβ deposition. Thus, Aβ stimulated human monocytes generate IL-8, MCP-1, MIP-1α and MIP-1β in vitro (Smits et al. 2002), and microglia cultured from rapid autopsies of AD and non-demented patients revealed an increased expression of IL-8, MCP-1 and MIP-1α after experimental exposure to Aβ (Lue et al. 2001). Neuropathological studies have found MCP-1 (Ishizuka et al. 1997) and increased expression of CCR3 and CCR5 in reactive microglia (Xia et al. 1998). Supporting the hypothesis that astrocytes actively contribute to the inflammatory disease component, MIP-1β has been detected in reactive astrocytes nearby Aβ plaques (Xia et al. 1998).

More recently a direct modulation of neurotoxicity by chemokines has been proposed, thus modulation of the CX3CR1/CX3CL1 system has been shown to influence neuronal survival in rodent models of neurodegeneration. While in the SOD1 mouse model for ALS, the MPTP neurotoxin model of Parkinson's disease and in a model of generalized inflammation CX3CR1 knockout mice showed an increased neuronal loss (Cardona et al. 2006), the same transgenic knockout prevented neuron loss in an triple transgenic mouse model of AD (Fuhrmann et al. 2010).

Inflammatory cytokines

The cytokine class of inflammatory mediators is secreted by microglia and astrocytes surrounding β-amyloid neuritic plaques. Cytokines associated with AD include several interleukins (ILs), TNF-α and TGF-β along with several others. Their production is increased in inflammatory states and they function by regulating the intensity and duration of the immune response.

In astrocytes, IL-1β induces IL-6 production, stimulates iNOS activity (Rossi and Bianchini 1996), and induces the production of M-CSF (Frei et al. 1992; Aloisi et al. 1992; Thery et al. 1992). In addition, IL-1β enhances neuronal acetylcholinesterase activity, microglial activation and additional IL-1α production, with consequent astrocyte activation, and expression of the cytokine S100β by astrocytes, thereby establishing a self propagating cycle (Griffin 2000; Mrak and Griffin 2001). IL-6 promotes astrogliosis (Selmaj et al. 1990), activates microglia (Heyser et al. 1997), and stimulates the production of acute phase proteins (Castell et al. 1989). IL-6 knockout mice exhibit a facilitation of radial maze learning over 30 days and show a faster acquisition, suggesting a possible involvement of IL-6 in memory processes (Braida et al. 2004). TNF-α has both pro-apoptotic and anti-apoptotic effects. This proinflammatory cytokine accounts for most of the neurotoxic activity secreted by monocytes and microglia (Combs et al. 2001). On the other hand, TNF-α has been reported to have neuroprotective properties in the AD brain.

In addition to the general role of cytokines, AD-specific interactions of certain cytokines and chemokines with Aβ may be pathophysiologically relevant. For example, TNFα can regulate APP processing and Aβ production in vitro (Blasko et al. 1999). In turn, fibrillar Aβ has been reported to increase neurotoxic secretory products, proinflammatory cytokines and reactive oxygen species as evidenced by a various groups and publications. Cultured rat cortical glia exhibit elevated IL-6 mRNA after exposure to the carboxy-terminal 105 amino acids of APP (Chong 1997). IL-1, IL-6, TNF-α MIP-1α and MCP-1 increase in a dose-dependent manner after cultured microglia are incubated with Aβ (Floden and Combs 2006; Lindberg et al. 2005; Benveniste et al. 2001; Butovsky et al. 2005; Veerhuis et al. 2005; Hanisch 2002; Lue et al. 2001; Lee et al. 2002). Production of IL-6 and M-CSF by human neurons is reportedly stimulated by glycation endproduct-modified tau and Aβ. Additionally, Aβ is able to stimulate a NFκB-dependent pathway that is required for cytokine production (Combs et al. 2001). The production of interleukins and other cytokines and chemokines may also lead to microglial activation, astrogliosis, and further secretion of proinflammatory molecules and amyloid, thus perpetuating the cascade (Ho et al. 2005).

A second general category of cytokine action is manifested by inhibitory, anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1Ra), IL-4, IL-10 and TGF-β. Some of these are reportedly elevated in AD, consistent with induction of homeostatic mechanisms in neuroinflammation (Grammas and Ovase 2001; Rota et al. 2006; Szczepanik et al. 2001a). The presence of anti-inflammatory cytokines such as IL-4 and TGF-β could be beneficial, because they are able to inhibit CD40 and class II MHC by restricting their expression and activity (Benveniste et al. 2001). However, overexpression of TGFβ in transgenic mice leads to changes in the microvasculature, including age-related amyloid deposition (Wyss-Coray et al. 2000), reflecting the multi-functional nature of many cytokines.

Even so AD mouse models are widely used to study in vivo consequences APP overproduction, only a limited

number of studies have characterized neuroinflammatory changes in these animals. All but one study used APP695 transgenic mice (Tg2576), and results were controversial. Mehlhorn et al. (2000) analyzed APP695 transgenic animals from 2 to 14 months of age but failed to detect mRNA levels of several cytokines including IL-1-α/β, IL-6, IL-10, IL-12 and IFN-γ using ribonuclease protection assay. In the same study, IL-1-β-positive astrocytes were detected in close proximity to Aβ deposition, whereas immunohistochemistry for TNF-α, IL-1-α, IL-6, and MCP-1 was negative. In contrast, Sly and colleagues detected TNF-α mRNA as early as 6 month of age (Sly et al. 2001), and Abbas and colleagues detected IFN-γ and IL-12 mRNA and protein levels by in situ hybridization and immunohistochemistry in 9-month-old APP 695 transgenic mice (Abbas et al. 2002). Moreover, IL-1-β, TNF-α and IL-10 were found by immunohistochemistry in 12–13-month old animals (Benzing et al. 1999; Apelt and Schliebs 2001). In our own study, IL-1β as well as IL-6 were detectable in CD11b positive microglia of APPV717I transgenic mice as early as at 3 month of age. The variability of findings in the same transgenic mouse line is likely caused by different techniques employed and indicates the difficulty of assessing inflammatory changes in these animal models. Differences between various APP mouse models, instead, may arise from time dependent changes of Aβ secretion. In contrast to the above described animal models, nearly all cytokines that have been studied, including IL-1α/β, IL-6, TNF-α, IL-8, and TGF-β, seem to be upregulated in AD compared to healthy age-matched individuals.

In addition to these primarily immunohistological evaluations, an association of AD with several polymorphisms of proinflammatory genes has been described, including IL-1 (Nicoll et al. 2000), IL-6 (Papassotiropoulos et al. 1999), TNF-α (McCusker et al. 2001; Perry et al. 2001a), and α1-antichymotrypsin, an acute phase protein (Kamboh et al. 1995). However, none of the various members of the interleukin cytokine family that are associated with AD actually map to chromosomal regions with evidence of genetic linkage (Tanzi and Bertram 2005). Thus, although inflammation and the upregulation of inflammatory mediators like the interleukins are regularly observed in AD brain, it appears less likely that variation at the genomic level of these proteins makes a large contribution to AD risk in general. An update on the genetic information can be found under http://www.alzgene.org

Cyclooxygenase and prostanoids

The two isoforms of cyclooxygenases, the mainly constitutively expressed COX-1 and the inducible COX-2, catalyze key steps of prostanoid synthesis in mammalian cells (O'Banion 1999). Downstream of both COX-1 and COX-2, several other enzymes regulate the generation of a whole spectrum of prostanoids, some of which may be neuroprotective and others neurotoxic. Thus, the composition and proportion of all prostanoids together may actually determine whether the activity of COX enzymes is beneficial or detrimental.

In vitro, LPS activated microglial cells and IL-1β-stimulated astroglial cells are capable of synthesizing COX-2 (O'Banion et al. 1996; Bauer et al. 1997). In contrast to peripheral monocytes, cultured rat microglia cells do not synthesize COX-2 in response to IL-1 or IL-6 (Bauer et al. 1997), suggesting that COX-2 regulation differs between CNS and peripheral cells. In rat microglial cell cultures, the major enzymatic product of COX-2 appears to be prostaglandin E_2 (PGE_2). Because PGE_2 itself is able to induce COX-2 in microglial cells (Minghetti et al. 1997), an autocrine or paracrine amplification of COX-2 in microglial cells and perhaps other cell types is possible. PGE_2 acts on four different receptors: EP1–EP4 (Narumiya et al. 1999). EP1 and EP2 receptors have been detected in cultured microglia, while EP3 receptors are also present in activated microglia in vivo (Slawik et al. 2004). Microglial EP2 receptors inhibit phagocytosis and enhance neurotoxic activities of microglia (Shie et al. 2005a; Shie et al. 2005b). In cultured rat and human astrocytes, EP2 and EP4 receptors are present and may potentiate glial cytokine production (Fiebich et al. 2001). PGE_2 may also act on the neuronal EP2 receptor, which is involved in apoptosis, although investigations of the role of neuronal EP2 activation on neuronal cell death have yielded conflicting results and suggest a neuroprotective role of neuronal EP2 stimulation under several pathophysiological circumstances (Bilak et al. 2004; Lee et al. 2004; McCullough et al. 2004; Takadera et al. 2004). Indeed, deletion of EP2 in a double transgenic AD mouse model led to decreased oxidative stress and amyloid burden (Liang et al. 2005). In conclusion, neuronal and glial secretion of PGE_2 may impair phagocytotic clearance of Aβ by binding to the microglia EP2 receptor and enhancing microglial toxicity. However, the role of PGE_2 in neurodegeneration may be far more complex due to the presence of other EP receptor subtypes on microglial cells and the effects of PGE_2 on other cell types. Neuronal death elicited by excitotoxins is elevated in transgenic animals with high expression of COX-2, suggesting that COX-2 expression may further interact with other pathogenetic mechanisms (Kelley et al. 1999).

COX-2 is upregulated in many inflammatory disorders (Dubois et al. 1998). However, data about COX-2 expression in AD brain are conflicting. Several investigators have detected increased levels of COX-2 mRNA and protein staining in AD tissue (Ho et al. 2001; Kitamura et al. 1999; Oka and Takashima 1997; Yasojima et al. 1999a) while others have found decreased COX-2 expression,

particularly in late stage AD (O'Banion et al. 1997; Yermakova and O'Banion 2001; Hoozemans et al. 2004). In addition to differences in tissue sources and methodologies employed, a well controlled post mortem study indicated a higher variability of COX-2 mRNA in the brains of AD patients compared to age matched controls (Lukiw and Bazan 1997). Furthermore, COX-2 expression is not restricted to microglial or astroglial cells in AD brain; indeed it is predominantly observed in neurons of AD brains and age matched controls (O'Banion et al. 1997).

Interestingly, COX-1 is prominently expressed by microglia in rodent and human brain (Yermakova et al. 1999; Hoozemans et al. 2001) and appears to be modestly elevated in AD brain (Yermakova et al. 1999). Whether microglial COX-1 contributes to neuroinflammation in AD has not been established; however, COX-1 activity has been linked to PGE_2 production in several experimental models of acute brain injury (Candelario-Jalil et al. 2003; Moore et al. 2005).

NO synthase, nitric oxide and free radicals

NO is a gaseous free radical, which is generated through the conversion of L-arginine to L-citrulline by enzymes of the nitric oxide synthase family (Bredt and Snyder 1990). Ca^{2+}/calmodulin-dependent constitutive isoforms are present in neuronal and endothelial cells, and produce NO in a highly regulated manner. iNOS is rapidly expressed in macrophages, microglia and astrocytes upon stimulation with lipopolysaccharide (LPS) or several cytokines (Galea et al. 1992; Corradin et al. 1993; Simmons and Murphy 1992; Stuehr and Marletta 1985). This isoform produces large amounts of NO in a Ca^{2+}-independent manner for prolonged periods of time. NO generated by iNOS is cytotoxic for invading microorganisms and tumor cells (Moncada et al. 1992). However, induction of iNOS may also have deleterious consequences for the host since vasodilatation, organ dysfunction and septic shock are partly mediated by an overproduction of NO (Thiemermann 1994). The consequences of iNOS induction in glial cells, however, seem to depend on a variety of factors including the type of cell cultures used. Both deleterious effects on neurons and unaffected neuronal viability after iNOS induction in mixed glial-neuronal cultures have been reported (Chao et al. 1996; Dawson et al. 1994; Demerle-Pallardy et al. 1993; Skaper et al. 1995). Importantly, iNOS expression and NO generation have been described in several brain pathologies including demyelinating diseases (Willenborg et al. 1999a, b), cerebral ischemia (del Zoppo et al. 2000), AIDS dementia (Hori et al. 1999), amyotrophic lateral sclerosis (Almer et al. 1999), and AD (Vodovotz et al. 1996; Wallace et al. 1997; Weldon et al. 1998; Lee et al. 1999; Heneka et al. 2001).

In addition to glial iNOS, neuronal iNOS may impact neurological disorders. For example, Vodovotz et al. (1996) reported that NFT-bearing neurons in affected brain regions of patients suffering from AD express iNOS. Further support for a role for iNOS in the inflammatory pathomechanisms involved in AD, is provided by reports of increased nitrotyrosine staining in AD brains, indicating sustained exposure and oxidative damage by peroxynitrite, an intermediate NO reaction product (Smith et al. 1997). In addition to NFTs and SPs, eosinophilic rod-like inclusions (Hirano bodies) are observed in AD brains and iNOS-immunoreactivity has been detected in association with Hirano bodies in the pyramidal layer of the CA1 region of the hippocampus and to a lesser extent in the stratum lacunosum (Lee et al. 1999). In that study, control brains showed only occasional iNOS-positive staining associated with rare Hirano bodies, while other studies, as well as our own experiments, failed to detect iNOS in control brains (MTH, unpublished observations).

To further characterize the pathway involved in neuronal iNOS expression in AD, we investigated expression of the enzyme argininosuccinate synthetase (AS) and its possible colocalization with iNOS in AD brain (Heneka et al. 2001). AS is the rate limiting enzyme in the metabolic pathway that recycles the iNOS substrate L-arginine from its catalytic byproduct L-citrulline. Several brain areas of AD patients showed a marked increase in AS expression in neurons and GFAP-positive astrocytes. Occasionally, AS expression was also detected in CD 68-positive activated microglia cells. Expression of AS was colocalized with iNOS immunoreactivity in neurons and glia. These results suggest that neurons and glial cells in AD not only express iNOS, but also AS. Because an adequate supply of L-arginine is indispensable for long-term NO generation by iNOS, coinduction of AS could enable cells to sustain NO generation, which could subsequently damage the iNOS expressing neurons as well as surrounding tissue.

Inflammation-permissive factors in AD

Occurrence and deposition of aggregated, misfolded or phosphorylated proteins may play the pivotal role for the induction and ongoing stimulation of inflammation in the AD brain. However, since some of these proteins may well occur in the normal aging brain without evoking such a dramatic immune response, it may be hypothesized that several other changes in AD are facilitating inflammation. Loss of aminergic brains stem nuclei, such as the locus ceruleus and the nucleus basalis of Meynert may result in an impaired control of neuroinflammation in the AD brain.

Locus ceruleus cell death

The locus ceruleus (LC) is located in the pontine tegmentum and serves as the main subcortical site for the synthesis of noradrenaline (NA) (Freedman et al. 1975). Ascending noradrenergic axons of the dorsal portion of the LC preferentially project to the hippocampus, the frontal and entorhinal cortex and to a minor extent to various other brain regions. Neuronal cell death of aminergic brain stem nuclei such as the LC and the dorsal raphe nucleus is a well defined, very early feature of AD that was first described by Forno (1966) and later confirmed by several groups (Mann et al. 1980, 1982; Wilcock et al. 1988). In AD, the central and dorsal portion of the LC show the most extensive loss of cells (Marcyniuk et al. 1986). LC loss and the subsequent degeneration of ascending noradrenergic axons lead to decreased NA levels in the LC projection areas (Iversen et al. 1983) whereas adrenergic receptors are upregulated in response to noradrenergic deafferentiation (Kalaria et al. 1989).

Besides its role as a classical neurotransmitter, NA acts as a potent suppressor of inflammatory gene transcription within the brain (Feinstein et al. 2002b; Marien et al. 2004). LC loss and subsequently decreasing NA levels may therefore be permissive for inflammatory mechanisms, which are otherwise controlled by physiologically released NA. Specifically, NA has been shown to suppress the generation and secretion of several inflammatory molecules including microglial synthesis of TNF-α and astrocytic expression of class II antigens. Studies from ourselves and others show that NA can also inhibit LPS- and cytokine-dependent iNOS expression in astrocytes and microglial cells, mediated by the activation of β-adrenergic receptors, and increases in cAMP (Gavrilyuk et al. 2002).

Since the initial neuropathological description, a number of studies have also demonstrated significant correlation of LC cell death or decreased cortical NA levels with severity and duration of dementia in AD (German et al. 1992; Yates et al. 1983). Interestingly, LC loss correlates better with the clinical course of the disease and the severity of dementia than loss of the nucleus basalis of Meynert and perturbation of the cholinergic system (Zarow et al. 2003). It has been argued that LC degeneration may occur as a consequence of primary degenerative changes in the cortical projection areas. However, even aged APP transgenic mice that do show an intense Aβ plaque load do not reveal any significant reduction of LC cell numbers or cortical NA levels, suggesting that LC cell death occurs independently of Aβ deposition and not in response to neurodegenerative events in its projection areas. This assumption is further supported by a recent finding that shows significant LC degeneration even in patients suffering from mild cognitive impairment, a clinical phase widely regarded as a prestage of AD (Grudzien et al. 2007).

Experimentally induced noradrenergic depletion can be achieved by systemic treatment of animals with the selective noradrenergic neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine (DSP4) (Fritschy and Grzanna 1989). DSP4 causes widespread degeneration of LC axon terminals, decreased activity, and loss of LC neurons (Fritschy and Grzanna 1989; Olpe et al. 1983). Moreover DSP4 also impairs electrophysiological functions of remaining LC neurons (Magnuson et al. 1993) and NA depletion by DSP4 has been demonstrated to markedly increase neurodegeneration induced by *N*-metyhl-4 phenyl 1,2,3,6-tetrahydropyridine (Mavridis et al. 1991) and cerebral ischemia (Nishino et al. 1991).

Using a similar model we showed that DSP4 treatment of rats caused degeneration of noradrenergic projections and cell death of LC neurons, but did not affect substantia nigra neuron survival. Local application of Aβ into the rat cortex resulted in a greater and prolonged IL-1β expression in microglial cells in noradrenergic depleted animals as compared to controls with an intact NA system. Likewise expression levels of IL-6, iNOS and COX-2 were significantly increased in NA depleted cortices. Interestingly, iNOS expression was completely restricted to microglial cells in controls, whereas NA depleted animals showed widespread iNOS expression in pyramidal neurons (Heneka et al. 2002; Heneka et al. 2003), a phenomenon previously reported in AD brains (Heneka and Feinstein 2001). Noradrenergic depletion in APP transgenic mice led to a similar picture, with increased glial activation and amyloid deposition, as well as evidence of increased neurodegeneration (Heneka et al. 2006). Additional in vitro and in vivo experiments assessing microglial migration and phagocytosis found that NA, while suppressing inflammatory gene transcription, increased microglial migration and phagocytic capacity at the same time (Heneka et al. 2010). Of note, microglial migration and Aβ phagocytosis, which was impaired in response to induced NA deficiency, could be re-established by treatment of animals with the NA precursor L-threo-DOPS. This may indicate that NA serves as an important factor that switches activated microglial cells from a cytokine releasing phenotype to a more mobile and phagocyting one. Thus, LC loss and noradrenergic depletion of cortical and hippocampal areas may drive neuropathological and inflammatory changes into a vicious, sustained cycle in AD. In keeping with this, balancing the NA deficit in AD may actively interact with disease pathogenesis and may open a new therapeutic avenue.

Basal forebrain cell death

Similar to cell death observed in the locus ceruleus, the basal forebrain nucleus (Ncl. basalis of Meynert) degenerates in AD. The neuronal loss observed here is thought to be the major factor for the subsequent decrease of acetylcholine (ACh) in the cortical projection regions of its neurons. The hypothesis that the loss of ACh is permissive for neuroinflammatory events in cortical projection areas was suggested by finding that efferent stimulation of the vagus nerve decreases the release of TNF-α and various other proinflammatory mediators by macrophages of the gastrointestinal tract (Borovikova et al. 2000; Wang et al. 2003; Pavlov and Tracey 2005). This effect has been attributed to the presence of the $\alpha 7$ subunit of the ACh receptor. Interestingly, glial cells of the CNS such as astrocytes and microglia cells express the $\alpha 7$ subunit (Graham et al. 2003) and expression of the $\alpha 7$ subunit is increased in astrocytes derived from AD patients compared to age-matched controls (Teaktong et al. 2003).

Diabetes mellitus

Diabetes mellitus (DM) is characterized by either an impaired production of insulin due to primary islet cell death or by insulin resistance of normally insulin responsive cells. The latter form is often observed at later stages of life and termed non-insulin dependent DM (NIDDM), because most of these patients can achieve control over the blood glucose level without subcutaneous insulin administration. NIDDM often becomes apparent at a similar time as AD and is an established risk factor for the development of AD (Ristow 2004). While the connecting and underlying mechanisms are yet unclear, one may hypothesize that impaired immunological defences of NIDDM patients and frequent peripheral infections contribute to the course of AD. Specifically, frequent infections result in higher levels of circulating cytokines and bacterial cell wall components such as lipopolysaccharides. Animal experiments suggest that the peripheral administration of lipopolysaccharides can contribute and enhance existing brain inflammation especially within the hippocampus (Semmler et al. 2005; Weberpals et al. 2009), and ultimately lead to an increased rate of Aβ plaque deposition (Sheng et al. 2003). In addition, recent work by Fishel et al. (2005) demonstrated that mild hyperinsulinemia in humans provoked an increase in cytokines and prostanoids in the CSF, suggesting stimulation of inflammatory brain circuits in NIDDM.

Cytokine driven feedback mechanisms

Apart from self-propagation and direct cytopathic effect on neurons, cytokines may more directly contribute to AD related neurodegeneration. Thus, studies performed in transgenic animals suggest that cerebral amyloid deposition is increased under inflammatory conditions (Games et al. 1995; Guo et al. 2002). Moreover, these animals do not develop amyloid plaques unless inflammation is induced suggesting that inflammatory molecules either raise the susceptibility for Aβ deposition and aggregation or directly influence the APP processing pathway.

Several lines of evidence suggest that cytokines may promote Aβ formation, aggregation and deposition at multiple levels. For example, IL-1α has been implicated in the transformation of diffuse β-amyloid aggregates into β-amyloid plaques. Furthermore, when the amyloid plaque associated proteins α1-antichymotrypsin (ACT) or apolipoprotein E were added to preparations of synthetic Aβ peptide in vitro, increased polymerization of Aβ into amyloid filaments was observed (Ma et al. 1994). In addition, cytokines are able to transcriptionally upregulate BACE1 mRNA, protein and enzymatic activity (Sastre et al. 2003). BACE1 and presenilin-1 are key enzymes in neuronal Aβ formation since in their absence, Aβ synthesis is either abolished or considerably reduced (Walter et al. 2001). These results are in line with data concerning increased expression and activity of BACE1 in NT2 neurons exposed to oxidative stress (Tamagno et al. 2002), in experimental traumatic brain injury (Blasko et al. 2004), and in reactive astrocytes in chronic models of gliosis (Hartlage-Rubsamen et al. 2003). Prolonged cytokine treatment can also influence βAPP maturation and secretion in various cell types (Blasko et al. 2000; Blasko et al. 1999). Finally, cytokines have been shown to increase APP expression. For example, TGF-β treatment of human astrocytes markedly elevated APP mRNA levels, and also increased the half-life of APP message by at least fivefold (Amara et al. 1999). In addition, IL-1α and IL-1β increased APP synthesis by up to sixfold in primary human astrocytes and by 15-fold in human astrocytoma cells without changing the steady-state levels of APP mRNA (Rogers et al. 1999).

Cytokines may also be involved in NFT formation since chronic IL-1β release from implanted capsules led to phosphorylation of neurofilaments and increased phospho-tau immunoreactivity in the rat hippocampus (Sheng et al. 2000). Similar studies have been reported in cortical neuron cultures (Li et al. 2003). The possibility that IL-1 represents a link between Aβ formation with consequent microglial activation and tau phosphorylation was recently supported by work in the triple transgenic mouse model of AD. In these animals, tau phosphorylation appears to proceed amyloid deposition (Oddo et al. 2003, 2006). However, phosphorylated tau-epitopes were observed in young animals that were chronically treated with intraperitoneal LPS prior to the time that plaques appear

(Kitazawa et al. 2005). These changes in tau were associated with increased levels of IL-1β and appeared to result from activation of cdk5 kinase.

Functional and structural consequences of neuroinflammation in AD

Over the past decade, numerous lines of accumulating evidence have strongly implicated that neuroinflammation contributes to AD pathogenesis. Irregardless at which time point of the disease it occurs, it seems clear that once initiated, inflammatory pathomechanisms can affect the AD brain. Functional and structural consequences may be differentiated to understand the various levels at which inflammation may contribute to AD.

Inflammation triggered functional impairment

LTP represents a key element of memory formation and consolidation. Since several cytokines including TNF-α, IL-1β and IFN-γ are able to suppress hippocampal LTP, the presence of these inflammatory molecules alone may be sufficient to induce neuronal dysfunction without structurally affecting neurons (Tancredi et al. 1992; Tancredi et al. 2000; Murray and Lynch 1998). Similar to cytokines, immunostimulated and iNOS derived NO also impairs LTP (Wang et al. 2004). Since iNOS expression is a long-lasting event, surrounding neurons face a sustained production of high NO concentrations. Functional impairment by increased cytokine and nitric oxide production may be of special relevance for the early stages of AD, when patients present with mild cognitive decline, often at a time when brain MRI scans fail to detect any atrophy of cortical or limbic structures.

Structural damage

In addition to neuronal dysfunction, several inflammation-evoked molecules may directly exert cytotoxic effects on neurons as already described. Despite descriptions of paradoxical protective effects for some of these mediators such as C5a (Osaka et al. 1999; Pasinetti et al. 1996; Tocco et al. 1997) or TNF-α (Feuerstein et al. 1994; Barger et al. 1995), the majority of experiments suggest that cytokines contribute to neuronal cell death. Further support for a more cytopathic role of these molecules comes from transgenic animal experiments showing that mice expressing these inflammatory proteins under brain-specific promoters invariably exhibit profound pathologic changes (Probert et al. 1995; Stalder et al. 1998; Wyss-Coray et al. 1997).

In addition to cytokine and complement generation, microglia and astrocytes may contribute by other means to neurodegeneration: While resting glial cells serve as an important source of various trophic factors such as GDNF, BDNF and others, chronic inflammatory activation may decrease the generation and release of these factors. It has therefore been suggested that inflammatory activation also leads to a significant trophic factor withdrawal which further contributes to neurodegeneration (Nagatsu and Sawada 2005).

Excitotoxic mechanisms significantly contribute to the neuronal loss in AD (Barger et al. 1993; Hensley et al. 1994; Smithswintosky et al. 1994). It is important to note, that the cytotoxic effects of iNOS derived NO and several proinflammatory molecules are not simply additive but potentiate NMDA or kainate induced excitotoxicity (Hewett et al. 1994; Morimoto et al. 2002).

Anti-inflammatory treatment strategies

Neuroinflammatory changes may even occur at early stages in the AD brain and significantly contribute to the pathogenesis of the disease. This raises the question whether therapeutic strategies can be developed which successfully target the ongoing inflammation.

NSAIDs as preventive treatment for AD

Epidemiological studies have suggested a beneficial effect of non-steroidal anti-inflammatory drugs (NSAIDs) in AD (Rogers et al. 1993; McGeer et al. 1996; Anthony et al. 2000; Breitner et al. 1995; Breitner 1996; Szekely et al. 2004; Stewart et al. 1997; Beard et al. 1998; In t'Veld et al. 2001). In particular, long-term NSAID therapy seem to delay the onset and progression of the disease, reduced symptomatic severity, and significantly slowed the rate of cognitive impairment (Rich et al. 1995), and at least one early treatment study with indomethacin suggested modest benefit in a small number of AD patients (Rogers et al. 1993). The epidemiological literature suggests an association between treatment duration and response for NSAIDs in preventing AD, with at least 2 years of exposure necessary to obtain full benefit (Breitner and Zandi 2001). Thus, the benefit may be greater the longer NSAIDs are taken (Etminan et al. 2003). Studies from aged controls and post mortem AD patients, both on reported NSAID medication, show that long-term NSAID therapy reduces the degree of plaque associated inflammation (Mackenzie and Munoz 1998; Mackenzie 2001; Alafuzoff et al. 2000). Nevertheless, these beneficial effects are limited to certain NSAIDS since naproxen and celecoxib were not able to modify AD (ADAPT Research Group et al. 2007).

Some of these beneficial effects have been further investigated in animal studies using APP overexpressing mice that display amyloid as well as inflammatory components of the disease. Several studies have demonstrated

that the NSAID ibuprofen acts to reduce astrocyte and microglial activation and cytokine production in APP transgenic mice (Lim et al. 2000; Yan et al. 2003; Heneka et al. 2005b). Six-month treatment with NSAIDs in Tg2576 significantly delayed AD symptoms, including a decrease of 40–50% in amyloid deposition (Lim et al. 2000) and improved performance in behavioral tasks (Lim et al. 2001). This effect was also observed in a short-term administration of a subset of NSAIDs to young Tg2576 APP mice, which lowered the soluble levels of $A\beta_{1-42}$ (Weggen et al. 2001; Eriksen et al. 2003). Moreover, treatment in the same mice with Meclofen, S-flurbiprofen or indomethacin reduced $A\beta_{1-42}$ levels (Eriksen et al. 2003). Additionally, it has been shown that long-term treatment with ibuprofen and indomethacin significantly decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in both cortex and hippocampus of APP transgenic (Tg2576) mice (Yan et al. 2003; Sung et al. 2004).

To date, the underlying mechanisms by which NSAIDs prevent AD are unclear, however evidence for several mechanisms has been put forward and there is the distinct possibility that actions at multiple rather than a single level of AD relevant pathology account for the observed beneficial effects of NSAIDs. Potential mechanisms include:

(1) Protection against $A\beta$ aggregation: It has been shown that certain NSAIDs may alter the β-sheet conformation of $A\beta$ affecting the aggregation of $A\beta$ peptides in vitro (Agdeppa et al. 2003; Thomas et al. 2001). Another report indicates that NSAIDs could induce the expression of amyloid binding proteins such as transthyretin, subsequently decreasing $A\beta$ aggregation (Ray et al. 1998).
(2) Effect on amyloid precursor protein (APP processing: The protective effect of NSAIDs has been associated with decreased secretion of $A\beta$ peptides and soluble APP, although there is still debate about the molecular mechanism involved (Weggen et al. 2001; Sastre et al. 2003). In particular, a subset of NSAIDs appears to directly affect the generation of $A\beta_{1-42}$, which is the most amyloidogenic form of $A\beta$. This subset of NSAIDs was shown to shift the cleavage products of APP to shorter, less fibrillogenic forms, indicating that NSAIDs could have an allosteric inhibitory effect on γ-secretase by altering PS1 conformation (Lleo et al. 2004).
(3) Inhibition of an alternate pathway: Ibuprofen has been shown to reduce pro-amyloidogenic α1-antichymotrypsin, an effect likely mediated by decreasing IL-1β (Morihara et al. 2005).
(4) Inhibition of cyclooxygenases: The canonical targets of NSAIDs are COX-1 and -2. Prostaglandin E_2 levels are increased fivefold in the CSF of probable AD patients (Montine et al. 1999) and COX-2 products are associated with neurodegeneration as discussed above.
(5) Several NSAIDs target the peroxisome proliferator activated receptor γ (PPARγ) (Lehmann et al. 1997), a nuclear hormone receptor studied for more than a decade by endocrinologists for its ability to increase insulin sensitivity. As described in more detail below, some of the beneficial effects ascribed to NSAID medication may be mediated by PPARγ activation.
(6) Prevention of ectopic neuronal cell cycle events, which indicate early neuronal vulnerability along with reduction of microglial activation. Importantly, this effect was independent of any modulation of the APP processing or steady state levels of $A\beta_{1-40}$ or $A\beta_{1-42}$ (Varvel et al. 2009).

Despite these multiple mechanisms, recent clinical trials with selective COX-2 inhibitors and the mixed COX-1/COX-2 inhibitor naproxen have been uniformly disappointing (Reines et al. 2004; Thal et al. 2005). This suggests that pathological changes may be too far advanced by the time of clinical diagnosis. Alternatively, off-target effects of NSAIDs may be critical and not maximally achieved with the specific drugs used in recent trials. Along these lines, neither COX-2 inhibitors nor naproxen inhibit $A\beta_{1-42}$ generation in vitro or in vivo (Eriksen et al. 2003).

PPARγ as target for NSAIDs

Ibuprofen, indomethacin and naproxen are among the five most prescribed NSAIDs, which have potentially decreased the risk for AD (In t'Veld et al. 2001). Interestingly, they are all agonists of the PPARγ (Lehmann et al. 1997). PPARs represent ligand-activated transcription factors that belong to a nuclear receptor superfamily and two isoforms, i.e. PPARγ_1 (Kliewer et al. 1994) and PPARγ_2 (Tontonoz et al. 1994) are formed from the same gene by alternative mRNA splicing. PPARγ_2 is specifically expressed in adipose tissue and differs from PPARγ_1 by the presence of 30 additional N-terminal amino acids that confer a tissue-specific transactivation function. PPARγ_1 is the predominant, if not the only, isoform in all other tissues, including skeletal muscle and liver (Li et al. 2000).

PPARγ forms heterodimers with retinoid X receptors (RXR) (Tugwood et al. 1992) and upon ligand activation, the PPAR/RXR heterodimer recruits coactivators and binds to sequence-specific PPAR response elements (PPRE) present in the promoter region of several target genes. Alternatively, PPARγ can inhibit specific gene expression without direct binding to the gene promoter, since transrepression of several genes, i.e. iNOS and COX-2, is achieved in part by antagonizing the activities of transcription factors

STAT1, NF-κB and AP-1 (Li et al. 2000; Daynes and Jones 2002; Kelly et al. 2004; Heneka et al. 2003).

PPARγ is involved in several cellular functions, including control of glucose homeostasis, regulation of systemic insulin sensitivity, cell differentiation and cholesterol metabolism (Vamecq and Latruffe 1999; Patsouris et al. 2004). The PPARγ gene knockout animal is embryonic lethal, due to essential roles in adipose, kidney and placental development (Barak et al. 1999). A role in the regulation of immune and inflammatory responses was suggested by the findings that PPARγ is expressed in macrophages and that receptor activation results in the inhibition of various inflammatory events, such as the production of IL-1β, TNF-α, IL-6 and iNOS (Ricote et al. 1998). PPARγ also appears to be involved in proliferation and production of IL-2 by T-lymphocytes and IFN-γ expression in murine CD4 and CD8 cells (Cunard et al. 2004).

In the brain, several anti-inflammatory effects of NSAIDs may be in part mediated through the activation of PPARγ (Landreth and Heneka 2001), since it has been shown that PPARγ agonists protect neurons from cytokine-mediated death (Heneka et al. 1999). Combs et al. (2000) reported that PPARγ agonists, including ibuprofen, inhibited Aβ-mediated microglial activation and neurotoxicity using in vitro models. Similar anti-inflammatory effects of PPARγ agonists and ibuprofen were also observed following infusion of immunostimulants into rodent brain (Heneka et al. 2000). In line with these findings, recent studies have documented the salutary effects of PPARγ-agonists in animal models of multiple sclerosis (Feinstein et al. 2002a; Niino et al. 2001; Diab et al. 2004; Natarajan and Bright 2002) and Parkinson's disease (Dehmer et al. 2004; Breidert et al. 2002) and Amyotrophic lateral sclerosis (Schutz et al. 2005; Kiaei et al. 2005). The potent anti-inflammatory effects of PPARγ-agonists suggest that they may have beneficial effects in treating other CNS diseases with an inflammatory component.

PPARγ activation is achieved by binding to a specific receptor binding pocket by various endogenous and synthetic ligands (Lehmann et al. 1997; Yki-Jarvinen 2004). Thus, PPARγ is stimulated best by 9-HODE (hydroxyoctadeca-9Z,11E-dienoic acid, 13-HODE and 15-deoxy-Δ12,14-prostaglandin J_2 ($15dPGJ_2$, although there is now considerable controversy as to whether 15d-PGJ_2 is actually a biologically relevant PPARγ activator or the majority of its action is mediated by inhibition of IκB kinase (IKKα and subsequently NFκB activation (Rossi et al. 2000). However, it has been reported recently that 15d-PGJ_2 is produced in vivo, and in large quantities by macrophages in vitro. Synthetic PPARγ ligands are used for their potent antidiabetic effects. In the United States, two ligands of the thiazolidinedione (TZD class, rosiglitazone and pioglitazone, have been approved for the treatment of NIDDM. Both substances bind PPARγ with high affinity and enhance insulin-mediated glucose uptake by increasing insulin sensitivity. In addition to receptor mediated effects some of these substances exert anti-inflammatory effects independently of PPARγ activation (Chawla et al. 2001; Feinstein et al. 2005).

Two in vivo investigations on the effects of PPARγ activation in APP transgenic mice have been reported. An acute 7 day oral treatment of 10-month-old APPV717I mice with the PPARγ agonist pioglitazone or the NSAID ibuprofen resulted in a reduced number of activated microglia and reactive astrocytes in the hippocampus and frontal cortex (Heneka et al. 2005b). Drug treatment reduced expression of the proinflammatory enzymes COX-2 and iNOS and the levels of BACE1. The same mice presented decreased Aβ42 levels, while a non-statistically significant reduction of about 20–25% in Aβ40 levels was found. Furthermore, intracellular Aβ staining was reduced in mice treated with ibuprofen or pioglitazone, indicating that PPARγ activation is involved in the regulation of Aβ generation (Sastre et al. 2006). A different study indicated that treatment of 11-month-old Tg2576 mice overexpressing human APP with the NSAID ibuprofen and PPARγ agonist pioglitazone for 16 weeks, only modestly reduced SDS-soluble Aβ levels and did not affect amyloid plaque burden (Yan et al. 2003). Since only 20% of pioglitazone crosses the blood brain barrier, and the study by Heneka et al. utilized twice the concentration used by Yan and colleagues, the observed difference may be explained by the drug concentrations applied.

All PPARs have been shown to be present in the CNS and to exhibit both unique and overlapping patterns of expression in various areas and at different developmental stages. The levels of expression of PPARγ in post-mortem brain sections from AD patients have been examined (Sastre et al. 2006) and immunohistochemical assessment of frontal cortex revealed that PPARγ is expressed in astrocytes and neurons. It has previously been suggested that AD brains contain increased levels of PPARγ in the cytosolic fraction compared to healthy controls (Kitamura et al. 1999). These results are in contrast with our own analysis showing a 40% reduction of PPARγ protein levels in AD patients compared to controls. In addition, PPARγ protein levels and its binding to a PPRE in the BACE1 promoter were decreased in AD brains (Sastre et al. 2006). Combined, these findings point to a direct role of PPARγ in the regulation of BACE1 transcription and activity in AD, ultimately facilitating Aβ generation.

Conclusions and future directions

Increasing evidence suggests that inflammation significantly contributes to the pathogenesis of AD. The generation and secretion of proinflammatory mediators may interact at multiple levels with neurodegenerative mechanisms. Thus, several proinflammatory cytokines cannot only induce neuropathic mechanisms and thereby contribute to neuronal death, but are also able to influence classical neurodegenerative pathways such as APP processing. The concomitant release of anti-inflammatory mediators may partly antagonize this action ultimately contributing to the chronicity of the disease. Several AD specific mechanisms such as locus ceruleus and nucleus basalis Meynert cell death may facilitate the occurrence of neuroinflammation. Future studies need to determine the underlying mechanisms and means by which the course of the disease can be influenced. Additional information on how inflammatory mediators and excitotoxic factors potentiate their detrimental effects is required. Additionally, more information is needed regarding the extent to which inflammatory mediators functionally impair cognition and memory.

Clinically, novel approaches to visualize early neuroinflammation in the human brain are needed to improve the monitoring and control of therapeutic strategies that target inflammatory and other pathological mechanisms. Similarly, more insight in the role of genetic factors that transmit a disposition for the disease should improve the detection of people at risk to develop AD.

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Exhibit B

Review TRENDS in Neurosciences Vol.29 No.9

The brain as a target of inflammation: common pathways link inflammatory and neurodegenerative diseases

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Classical knowledge distinguishes between inflammatory and non-inflammatory diseases of the brain. Either the immune system acts on the CNS and initiates a damage cascade, as in autoimmune (e.g. multiple sclerosis) and infectious conditions, or the primary insult is not inflammation but ischemia or degeneration, as in stroke and Alzheimer's disease, respectively. However, as we review here, recent advances have blurred this distinction. On the one hand, the classical inflammatory diseases of the brain also exhibit profound and early neurodegenerative features – remarkably, it has been known for more than a century that neuronal damage is a key feature of multiple sclerosis pathology, yet this was neglected until very recently. On the other hand, immune mechanisms might set the pace of progressive CNS damage in primary neurodegeneration. Despite differing initial events, increasing evidence indicates that even in clinically heterogeneous diseases, there might be common immunological pathways that result in neurotoxicity and reveal targets for more efficient therapies.

Introduction

The immune response within the brain is limited or unusual, a condition that has led the brain to be described as an 'immune-privileged' organ (see Glossary). Immunocompetent cells regularly enter the brain with no apparent subsequent pathology [1], and locally acting microglial and dendritic cells have important immunological properties [2,3]. Inflammation occurs as a massive invasion of blood-borne immune cells during, for instance, local host defence upon bacterial or viral infection, or during harmful autoimmunity (see Glossary) such as multiple sclerosis (MS; see Glossary) where, in the absence of any specific infectious agent, the immune system attacks structures of the CNS. MS, the classical chronic inflammatory pathology of the CNS, leads to devastating disability in young adults and cannot yet be cured; neither can its progression be dramatically influenced. Anti-inflammatory therapies have some impact (Table 1), but these remain unsatisfactory. To explain this, we propose that established therapeutic concepts, primarily targeting the immune system outside the CNS, have so far neglected ongoing or even uncoupled neuronal injury within the CNS.

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Inflammation has also been identified as a relevant factor in primarily non-inflammatory CNS disorders. Here, the primary insult is not inflammatory but degenerative, metabolic or ischemic. This might be why any associated inflammatory glial responses in these diseases have often been classified as unspecific 'reactive gliosis' that deserves no further serious consideration. However, there is now increasing evidence that progressive CNS damage in primary neurodegeneration is controlled and promoted by immune mechanisms. Our understanding of these unexpected pathological features is still at a very early stage, although currently at its most advanced in stroke and Alzheimer's disease (AD; see Glossary). Here, we review current evidence of neurodegeneration arising in the inflamed brain, and inflammatory processes that develop upon degeneration or injury of the CNS. In light of these findings, we postulate that both classical inflammatory and classical neurodegenerative or injury pathologies of the CNS share common molecular mechanisms, which bring together these previously distinct areas of neuroscience.

A lesson to learn – a paradigm shift for MS

In MS, the description of demyelination and relative preservation of axons (Box 1), and the ability to induce a similar pathology in animals by immunization with myelin components, have guided the research focus towards the myelin sheath and the oligodendrocyte as central and specific targets. These are found predominantly in the white matter, which lacks neuronal cell bodies. However, this perspective fails to take into account earlier pathological findings that axons are not preserved, and that the grey matter (i.e. the cortex and deep brain nuclei) is also involved. Subsequently, the low sensitivity of conventional magnetic resonance imaging (MRI) to grey matter changes such as plaques or atrophy, together with the higher frequency of contrast enhancement in the white matter, might help to explain the lack of attention towards involvement of neurons in the disease. More than a century after the first histopathological evidence, several groups rediscovered the importance of neuronal damage in MS [4–6]. Brains of patients show early axonal pathology that correlates with immune cell infiltration [5], 'black holes' as a sign for complete tissue loss in MRI, and death of neuronal cell bodies [7]. Cortical lesions were reported in 26% of patients [8], in line with MRI data on focal cortical thinning and widespread grey matter involvement,

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Glossary

Adrenoleukodystrophy (ALD): a rapidly progressive X-linked inherited recessive disorder characterized by the accumulation of saturated very long chain fatty acids (VLCFA) predominantly in the white matter of the brain and the adrenal glands. ALD is caused by disturbed breakdown of VLCFA due to mutations of the ABCD1 gene family, which encodes ATP-binding cassette (ABC) transporters. The disease leads to MS-like symptoms, but it does so with a very rapid course, and is usually fatal in childhood.

Alzheimer's disease (AD): a disease characterized by a loss of mental functions (dementia) and in later stages motor disability due to damage to brain tissue. The German physician Alzheimer discovered and characterized both the clinical and histopathological features of this disease in 1907. AD is the most common cause of dementia, rarely occurring before the age of 50. The disease takes from a few months to four or five years to progress to complete loss of intellectual function. Its exact aetiology is still unknown, but environmental and genetic factors are thought to contribute.

Autoimmunity: immunological recognition of self. The consequences of this process can be either harmful or beneficial. On the one hand, the body's immune system might attack and harm its own tissues, leading to an autoimmune disease. On the other hand, the immune system might contribute to the restoration of tissue integrity.

Huntington's disease (HD): first described by the American family practitioner Huntington in 1872 as an inherited degenerative brain disease characterized by intellectual decline and involuntary movement of limbs. The onset of the ultimately fatal disease occurs later in life and is associated with the death of a population of neurons in the basal ganglia of the brain. A trinucleotide repeat (CAG repeat) of variable but abnormal length is specifically related to HD and to the protein 'huntingtin', the role of which is still not entirely clear.

Immune-privileged organ: inflammation is the first response of the immune system to danger signals evoked by infection or irritation. The unique ability to distinguish between self and non-self enables the immune system to identify and defeat invading infectious agents. The same mechanism is responsible for the rejection of transplants from another donor. However, since Van Dooremal's report in 1873, it has been known that the brain shows relative tolerance to various grafts, with prolonged graft survival. Indeed, Medawar noted 1948 that grafts in the brain 'cannot elicit an immune response', but are rejected from the brain upon peripheral immunisation. In 1953, Billingham and Boswell referred to this situation as an 'immunologically privileged status' of the brain, possibly indicating the evolutionary necessity to reduce certain immunological processes within particularly vulnerable tissues.

Meningitis: inflammation of the membranes (meninges) covering the brain upon bacterial and viral infection. Most prominent in the case of bacterial meningitis, clinical symptoms include a horrible headache, fever, malaise and pain when moving the neck. Encephalitis (as sign for involvement of the brain parenchyma) can complicate infectious meningitis (i.e. meningoencephalitis).

Multiple sclerosis (MS): the most common chronic inflammatory disease of the CNS and the major cause of neurological disability in young adults in developed countries. The clinical manifestation is characterized by recurrent episodes of neurological deficits, such as loss of vision or paralysis, followed by periods of remission. The first concise disease study was undertaken by the French physician Charcot in 1868 who described the clinical heterogeneous picture with the corresponding neuropathology, characterized by multifocal inflammatory brain and spinal cord lesions of different stages (Box 1). Within these lesions, activated immune cells, particularly T lymphocytes and macrophages, can be detected. Despite intensive efforts, no infectious agent causing the immune attack has been identified so far. Therefore, MS is considered to be an autoimmune disease of the CNS. In MS, physicians use magnetic resonance imaging (MRI) of the brain, spinal cord and optic nerves to localize the lesions and to measure progression of the disease. The most commonly used model of MS is experimental autoimmune encephalomyelitis (EAE), which can be induced in susceptible animals (mostly rodents) by immunization with myelin components or transfer of activated myelin-specific T lymphocytes.

Parkinson's Disease (PD): a slowly progressive neurodegenerative disease of the substantia nigra, an area localized in the basal ganglia of the CNS. The first clinical description of the disease was published in 1817 by the British physician Parkinson. He named the disease shaking palsy and described characteristic muscular tremor, slowing of movement, partial facial paralysis, peculiarity of gait and posture, and weakness. Typically, when patients present with first clinical signs, the vast majority of nigral neurons that synthesize dopamine have perished. In most cases, the causes of the disease remain unknown (idiopathic PD). Symptoms of PD are observed in humans who abuse the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Stroke: occurs when the blood flow to a part of the brain is suddenly blocked by occlusion (ischemic stroke – responsible for ~90% of strokes). The acute death of brain cells is caused by reduced delivery of oxygen. Within the ischemic core zone, most of the affected cells are neurons, because they show the highest vulnerability to oxygen deprivation. Surrounding the ischemic core zone, there is an area of moderately ischemic brain tissue called penumbra (peri-infarct zone) where surviving cells are exposed to secondary deleterious phenomena, such as excitotoxicity, spreading depolarization and inflammation. Thus, the prime goal of neuroprotection is to rescue the penumbra. Clinically, depending on the part of the brain that is affected, strokes can result in weakness or paralysis of the arms, legs and/or facial muscles, loss of vision or speech, and impaired walking.

Wallerian degeneration: Axonal degeneration resulting from proximal injury to the same axon, first described by the English physiologist Waller in 1850.

even at early stages [9,10]. Clinically, these alterations are reflected by frequent cognitive impairment [11], electroencephalographic abnormalities in 20–60% of MS patients [12], and as much as a tenfold increase in frequency of epileptic seizures [13]. This appreciation that neuronal damage significantly contributes to the disease has prompted a detailed analysis of the crosstalk between the immune and nervous systems within the CNS.

Primary immune attack targeting the CNS

Mechanisms of collateral neuronal damage

In primary neuroinflammatory diseases, activated immune cells are attracted by the presence of their target within the CNS compartment. Depending on the time course and the target in question, which can be the myelin sheath in MS, or infectious agents such as bacteria in meningitis (see Glossary), the inflammatory infiltrate consists of macrophages, microglia, neutrophil granulocytes, T cells and B cells. The invading immune cells battle effectively against their target, thus inducing, for instance, a loss of myelin or the death of harmful infectious agents. However, even though the primary target of the immune system might not be neuronal, neurons are nevertheless severely injured during this attack. This has been demonstrated for MS, where MRI investigation of normal appearing white matter shows extensive axonal pathology that is interpreted as Wallerian degeneration (see Glossary) and taken to indicate early damage of neuronal structures [14]. The decline of *N*-acetyl-aspartate (NAA) levels as a sign for neuronal destruction, the increase in atrophy, and the accumulation of axonal loss, all correlate with disability [15]. Similarly, survivors of bacterial meningitis exhibit extensive neuronal death in the hippocampus [16], despite state-of-the-art antibiotic therapy. Neuronal damage in this sensitive area responsible for learning and memory might explain the diverse neurological symptoms of survivors, including seizures, motor deficits, hearing loss and cognitive impairment. Cortical neuronal apoptosis is also a key feature in HIV encephalopathy, which is caused by CNS infection with HIV-1 and characterized by the presence of infected macrophages in the brain [17]. Because neurons themselves are not infected, and neuronal damage shows no correlation with productive HIV infection, an indirect mechanism of neuronal damage has been suggested [17].

Hence, unexpected 'collateral' damage to neurons occurs in classical neuroinflammatory diseases. The simplest explanation for this seems to be that invading immune cells directly interact with neurons, causing them harm.

Table 1. Successful immunotherapies in inflammatory neurodegeneration[a]

Approach	Substance or class	Disease, and stage of immunotherapy success	Refs
Broad immunosuppression	Bone marrow transplantation	MS, clinical trials	[79]
		ALD, established therapy	[80]
	Corticosteroids	MS, established therapy	[81]
		Bacterial meningitis, established therapy	[82]
NSAID	Combined COX1/COX2 inhibitors	AD, epidemiological studies and transgenic mouse models	[83–85]
		PD, epidemiological studies and MPTP mouse model	[86,87]
	COX2 inhibitors ('COXIBs')	AD, transgenic mouse model	[88]
		PD, MPTP mouse model	[87]
Selective immunomodulators	Glatiramer acetate	MS, established therapy and EAE mouse model	[89]
		AD, transgenic mouse model	[90]
		PD, MPTP mouse model	[91]
	Flavanoids	MS, EAE mouse model	[92,93]
		AD, transgenic mouse model	[94]
		PD, MPTP mouse model	[95]
	IVIg	MS, phase II trial (relapsing–remitting MS) and EAE mouse model	[96,97]
		AD, pilot trial	[98]
	Statins	MS, pilot trial and EAE mouse model	[99–101]
		AD, epidemiological studies	[102]
		PD, MPTP mouse model	[103]
		ALD, pilot trial	[104]
	PPARγ agonists	MS, EAE mouse model	[105]
		AD, transgenic mouse model	[85]
		PD, MPTP mouse model	[106]
	Vaccination	AD, pilot trials and transgenic mouse models	[63]
		PD, transgenic mouse model	[107]
		Stroke, mouse model	[108]
	Migration blockers	MS, pilot trials and EAE mouse model	[109]
		Stroke, mouse model	[110]
		Meningitis, rabbit model	[111]
	Minocycline	MS, pilot trials and EAE mouse model	[112,113]
		HD, transgenic mouse model	[114]
		Stroke, mouse model	[115]

[a]Abbreviations: AD, Alzheimer's disease; ALD, adrenoleukodystrophy; COX, cyclooxygenase; EAE, experimental autoimmune encephalomyelitis; HD, Huntington's disease; IVIg, intravenously applied polyclonal immunoglobulins; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; NSAID, non-steroidal anti-inflammatory drugs; PD, Parkinson's disease; PPARγ, peroxisome proliferator-activated receptor-γ.

License to kill – the affinity of activated immune cells to neurons

T cells. Such a 'fatal attraction' between invading immune cells and neurons had not been considered relevant or even possible, because (i) neurons do not normally express major histocompatibility complex (MHC) molecules, which are in principle required to make target cells accessible for immune cells, and (ii) cells invading the CNS in the course of neuroinflammatory diseases are not usually specific for neuronal antigens. However, recent studies have shown that T cells can, upon activation, gain the capacity to harm neurons *in vivo* and *in vitro*, independently of their antigen specificity [18,19]. Investigating the possibility of direct and rapid injury of the neuron itself, it has recently been shown using two-photon microscopy that encephalitogenic $CD4^+$ T lymphocytes [20] possess marked migratory capacities within the CNS parenchyma and directly interact with the soma and

Box 1. Histopathological evidence for neuronal injury in multiple sclerosis – a historical outline

1868: Charcot describes that MS primarily affects the white matter, and therein exclusively the myelin sheath, leaving the axons unaffected.
1880: Charcot attenuates his prior assumption of axonal preservation in MS and speculates that fixed neurological deficits might be linked to irreversible axonal damage.
1916: Dawson publishes systematic studies on white and grey matter pathology. He confirms the existence of 'numerous alterations which axis cylinders undergo' and mentions different stages of axonal injury, including 'spindle-shaped swellings' and 'disintegration'. He states: 'It is now well recognised that the cortex is affected in disseminated sclerosis.' Subtle changes of cortical neurons such as 'an absence of the processes, a rounding of the cell body' and a 'gradually advancing atrophy' are interpreted as the first signs of degeneration. He notices that 'in a later stage such cells become wholly lost'. Remarkably, he detects the association of degenerating neurons with 'small nests of cells', possibly activated immune cells.
1936: Putnam is the first to use a quantitative approach in determining the extent of axonal damage, and detects an 'unmistakable decrease in number' of axons.
1962: Brownell and Hughes investigate macroscopically the distribution of cerebral plaques and recognize lesions occurring 'at the junction between cortex and white matter' or even purely within the grey matter.
1983: Raine notes that white matter plaques display a reduced density of surviving axons and suggests that axonal damage is not an uncommon event.
1997: Ferguson, Perry and colleagues show immunohistochemical evidence for the expression of amyloid precursor protein within acute MS lesions, indicative of axonal injury.
1998: Trapp and colleagues provide immunohistochemical evidence for structural transection of axons as a consistent feature of MS lesions and correlate this to the degree of inflammation.
2000: Kornek, Lassmann and colleagues quantify axonal damage and demonstrate the highest incidence of axonal destruction during active demyelination.
2001: Peterson, Trapp and colleagues detect apoptotic neurons and transected neurites in demyelinated cortical MS lesions.

processes of neurons, partially leading to cell death [21] (Figure 1a). So how might T cells induce neuronal apoptosis? There is increasing evidence for the crucial contribution of the death ligand tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) as a T-cell-associated effector molecule. Indeed, TRAIL expressed by $CD4^+$ T cells induces collateral death of neurons in the inflamed brain and promotes experimental autoimmune encephalomyelitis (EAE) [22]. This observation has direct relevance for MS, because early immunohistochemical studies described the predominance of $CD4^+$ T cells in active MS lesions [23]. Previous

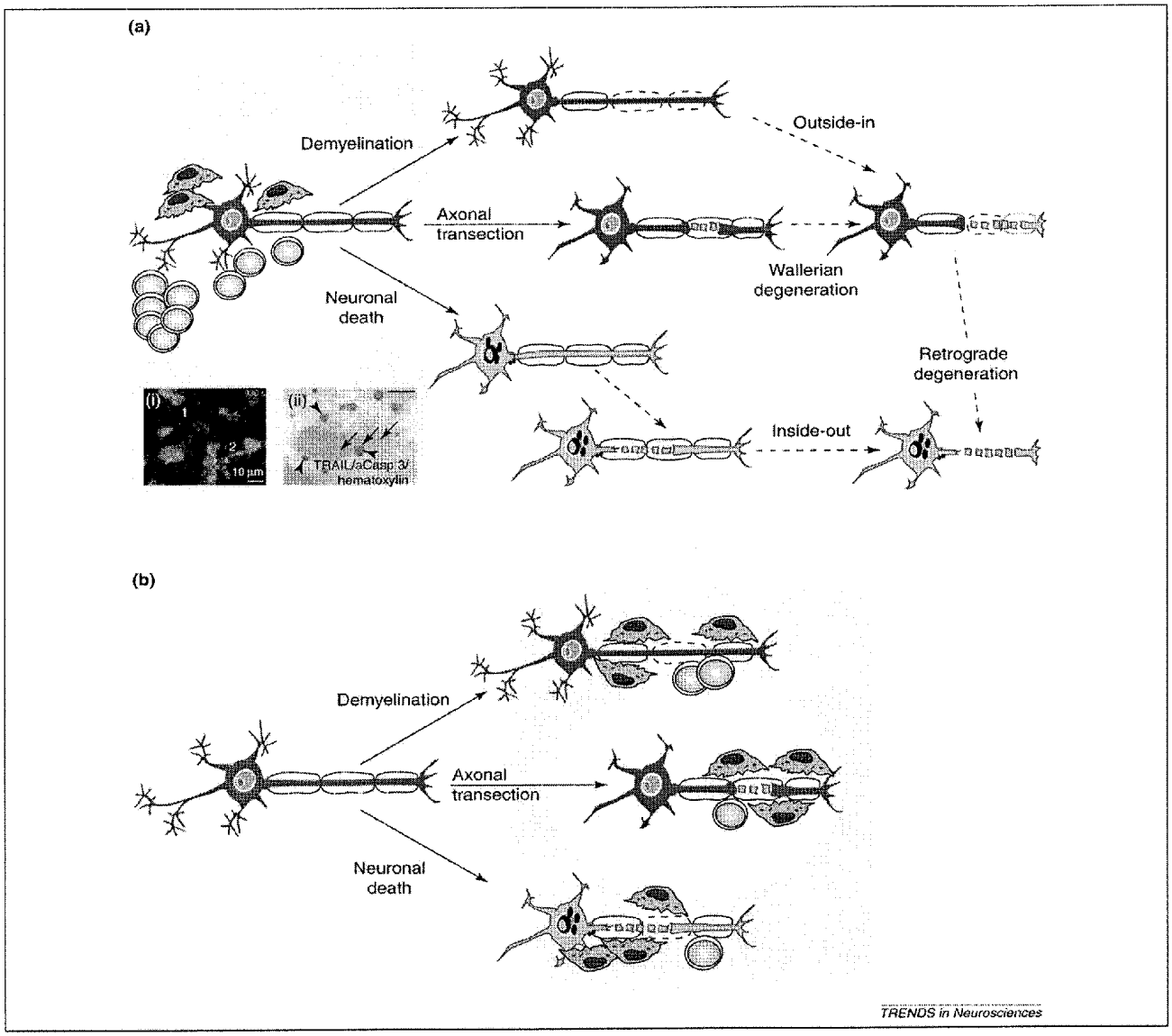

Figure 1. Possible sequence of inflammatory brain tissue injury in the course of neurodegeneration. **(a)** In primary inflammatory CNS diseases (e.g. MS), activated immune cells, mainly macrophages, microglia and lymphocytes (orange), attack a common antigen of the brain, typically parts of the myelin sheath. During the immune attack (indicated by the shaded background), demyelination, axonal transection or direct death of the neuron can occur. In the course of the disease, demyelination can lead to secondary axonal transection (outside-in damage), or death of the axon or neuron might result in secondary demyelination (inside-out damage). Some of these mechanisms might also be part of the pathology of diseases such as AD. Broken arrows indicate indirect degeneration processes. Inset: T lymphocytes possess marked capacities to migrate within the CNS parenchyma and to interact with neurons. (i) Direct contact of T lymphocytes (red) with hippocampal neurons (green) in living brain tissue is shown by two-photon microscopy (see corresponding movie at http://www.jneurosci.org/content/vol24/issue10/images/data/2458/DC1/Movie_3B_JN-RM-4703-03.mpg). A T-cell-induced transient increase in neuronal Ca^{2+} concentration occurs after contact with cell body (1) or neurite (2). Reproduced, with permission, from Ref. [21] © (2004) the Society for Neuroscience. (ii) Evidence for lymphocyte-mediated neuronal apoptosis in the MS animal model EAE: a light micrograph of TRAIL-expressing immune cells (dark blue–black) in the vicinity of, and in some cases (arrowheads) in direct contact with, a brainstem neuron in the early phase of apoptosis [i.e. exhibiting activated caspase 3 staining (aCasp 3; brown signal; arrows) in its cytoplasm; its nucleus is stained using haematoxylin]. Scale bar, 20 µm. Reproduced, with permission, from Ref. [22] © (2005) Elsevier. **(b)** In primary degenerative CNS diseases such as ALD or AD, and in stroke, the initial event is a non-immune-mediated injury of the CNS. However, immune cells, predominantly local microglia and macrophages, and under certain conditions lymphocytes (orange), become involved during the degenerative processes and elicit a secondary inflammatory reaction, which sets the pace of progressive neuronal injury. This sequence of events might also occur at certain stages of MS or in some MS patients.

reports on clonally expanded CD8+ T cells from MS biopsies, $CD8^+$ T cells associated with axons in the lesions, and *in vitro* cultured neurons rendered vulnerable to $CD8^+$ T cell-mediated death [24] all point to the significance of these cells. However, one recent EAE study in β2-microglobulin-knockout mice lacking mature $CD8^+$ T cells showed an aggravated disease course with increased axonal damage [25]. Thus, this T-cell subset might even have important anti-inflammatory functions *in vivo* [26–28].

Macrophages and microglia. Even more prominent than T cells are the other key players involved in classical neuroinflammatory diseases, the microglia [29] – resident glia partially derived from circulating monocytes [30] that display high sensitivity to different kinds of CNS injury and take on the morphology of activated macrophages (Figure 1a). In the case of MS, an insidious process was recently found to link inflammatory demyelination to neuronal damage. This process is mediated by the oxidative myelin breakdown product 7-ketocholesterol (7-KC) [31], which accumulates in the brains of MS patients and significantly correlates with clinical disability and neuronal death in EAE. Although 7-KC has no direct detrimental effects on neurons, it does induce a migratory and neurotoxic phenotype in microglia [31]. Activated microglia in the cortex of MS patients physically associate with neuronal perikarya and proximal dendrites [7]. A similar association of microglia – stimulated by HIV envelope coat proteins – and apoptotic neurons in the cortex has been described for HIV encephalopathy [17]. Similarly, infiltrating macrophages, microglia and neutrophil granulocytes are thought to mediate neuronal damage in meningitis [16,32]. Here, moribund bacteria release unmethylated CG-dinucleotide-motif-rich DNA, which in turn strongly induces microglia-mediated neurotoxicity via the toll-like receptor-9 [33]. Indeed, adjuvant anti-inflammatory treatment with corticosteroids, or blocking of leukocyte invasion into the CSF, reduces neurological deficits in meningitis (Table 1).

Thus, activated immune cells have the intriguing capacity to harm neurons directly – irrespective of the antigen specificity. Weighing the contribution of the different immune cells, one might argue that, in contrast to microglia, the direct neurotoxic effect of T cells is not permanent but, rather, fluctuates and is restricted to the acute phase of invasion into the brain, because T-cell survival within the brain parenchyma is limited [34]. However, regarding chronic inflammatory diseases, their potent capacity to initiate a full-blown immune response, including the activation of microglia, and to cruise the brain tissue, as demonstrated by two-photon microscopy, underlines their possible relevance in disease. Moreover, the attraction of microglia to neurons and their interactions might initially cause only subtle degenerative changes, such as neuritic beading (focal swellings in dendrites and axons), through NMDA receptor signalling [35]. Here, inflammation does not necessarily cause immediate neuronal death, but might gradually increase the susceptibility of neurons to degeneration at later stages.

Death by starvation – neurons wasting away

In MS, there is even evidence for non-inflammatory primary demyelination [36] and gradual neurodegeneration independently of inflammatory invasion [37]. Secondary axonal dysfunction or loss might occur owing to a lack of the support normally supplied by the oligodendrocytes [38]. A possible molecular candidate is ciliary neurotrophic factor (CNTF): EAE-affected knockout animals lacking CNTF show similar inflammation to wild types, but enhanced axonal pathology associated with vacuolar degeneration of the myelin sheaths [39]. Tsunoda, Fujinami and colleagues suggested that, apart from this 'outside-in' (i.e. myelin sheath to axon) pathology, an 'inside-out' pathology (Figure 1a) is supported by observations of secondary demyelination caused by virus-mediated neuronal and axonal injury [40]. Moreover, a lesion in the CNS – be it a neuronal injury [41–43] or oligodendroglial damage [44] – brings in its wake an immune response that might develop into autoimmunity under specific circumstances (Figure 1b). Such processes could indeed have a role early in MS [45], at certain stages of the pathology, or in certain patients [36,46]. Death of neuronal cell bodies in MS [7] might also result from a retrograde degeneration after axon pathology (Figure 1a). However, it should be noted that axonal injury does not necessarily lead to subsequent death of the cell body [47]. In fact, in the very early phase of MS, progressive atrophy of grey but not white matter has been detected [48]. These are remarkable findings, and even constitute evidence for an inflammation-independent, uncoupled insult to the neuron – a finding more commonly associated with classical neurodegenerative diseases. These considerations lead us to the question of whether and how non-inflammatory brain diseases are connected with and controlled by inflammatory processes.

Friend or foe? 'Secondary' inflammation in non-inflammatory brain diseases

The concept of 'secondary' inflammation in primarily non-inflammatory neurodegenerative disorders of the CNS, or stroke, stems from models in which non-inflammatory brain injury attracts leukocytes to denervated areas [41–43] (Figure 1b). In fact, it should be borne in mind that immune responses are not harmful *per se*, because they are an integral tool of the self-maintenance programme of an organism, enabling it to combat invading infectious agents and enhancing wound healing. A mild autoimmune reaction might even confer neuroprotection in certain models of CNS injury [49,50]. However, in the case of neurodegenerative brain diseases, several lines of evidence, including immune-based therapy studies, suggest that inflammation has a deleterious impact on ongoing pathology (Table 1). For example in adrenoleukodystrophy (ALD; see Glossary), in which inflammation is an epiphenomenon but crucially contributes to clinical severity [51], the initial brain insult originates exclusively from genetically disturbed peroxisomal oxidation of very long chain fatty acids. The cerebral lesions show massive breakdown of myelin and a perivascular cellular infiltrate of $CD4^+$ and $CD8^+$ T cells, and to a lesser extent of B cells and microglia. Bone marrow transplantation, initially aimed at correcting the altered metabolism of accumulating fatty acids by introducing normal non-mutant blood cells, has a beneficial impact on clinical deterioration and cerebral inflammation

without correcting the fatty acid levels (Table 1). The outcome of stroke also depends on activated microglia recruiting numerous potentially neurotoxic leukocytes [52–54]. Blocking the activity of key inflammatory mediators such as interleukin (IL)-1 reduces brain damage in ischemia and other neurological diseases [55]. In AD, and also Parkinson's disease (PD) and Huntington's disease (HD; see Glossary), there is no obvious accumulation of activated immune cells, in contrast to ALD or stroke. Nevertheless, the plethora of recent studies demonstrating the crucial contribution of the immune system (Tables 1 and 2) has prompted a radical paradigm shift for these diseases. This is particularly true for AD, where potent inflammatory molecules such as cytokines, chemokines and complement are detected in the CSF and in plaques from AD patients, and where there is known to be a genetic association with inflammatory molecules [56]. It is currently believed that discrete degenerative processes at the beginning of AD – such as the deposition of highly insoluble amyloid β (Aβ) and the accumulation of tau proteins – damage neurons and provide clear inflammatory stimuli to local microglia [57,58]. In this context, processes contributing to the downregulation of immune response in the CNS might be disturbed as part of the pathology [59]. Macrophages and microglia have been reported to be activated not only in autopsies of patients with idiopathic PD and AD [60] but also upon MPTP intoxication (see Glossary) of humans who thereafter suffer from a Parkinson syndrome [61]. Similarly, accumulation of reactive microglia in the direct vicinity of neurons that have huntingtin-positive nuclear inclusions was found at early and progressive stages in the HD brain [62]. In AD and PD, epidemiological studies revealed that systemic inflammation increases disease susceptibility, because unspecific anti-inflammatory treatment for other reasons – such as rheumatoid arthritis – lowers the risk of developing AD or PD. Interestingly, the induction of a specific immune response by vaccination with an Aβ segment markedly reduced pathology in different transgenic animal models of AD [63] and had an effect in patients, despite occasional induction of meningoencephalitis [63]. To take another example, it is striking that immunomodulatory therapy with glatiramer acetate, a myelin analogue approved for MS therapy, suppressed ongoing inflammation within the brain in experimental models of AD and PD (Table 1).

Thus, initially divergent events, such as ischemia, peroxysomal dysfunction or deposition of Aβ or α-synuclein, clearly initiate involvement of the immune system [57,64], which in turn interacts with the nervous system and ultimately sets the pace of progressive tissue damage in the CNS. The essential question now is whether such distinct clinical disorders as MS and AD share common mechanisms.

Shared molecular pathways

Which molecules are found in shared pathways of inflammatory neurodegeneration? Certainly the level of experimental evidence is not the same for all the diseases mentioned, because traditional paradigms have tended to focus attention on pure neurodegenerative or pure inflammatory mechanisms, neglecting an integrated view. However, among the list of factors comprising cytokines, death ligands, neurotransmitters, and other small molecules (Table 2), some favourites merit a closer look.

IL-1

A prominent role in different paradigms of neuronal damage has been suggested for the IL-1 family, which is now regarded as a key contributor to diverse neurodegenerative conditions, both acute (e.g. stroke) and chronic (e.g. AD) [55]. Expression of IL-1 is enhanced by soluble inflammatory mediators such as lipopolysaccharide, complement and prostaglandin E2, and also by molecular components of CNS cells such as the Ca^{2+}-binding protein S100 (which is released from injured astrocytes), Aβ (from AD plaques)

Table 2. Current evidence that effector mechanisms within the CNS contribute to inflammatory neurodegeneration[a]

Effectors	Disorder								Refs
	MS	Meningitis	AD	PD	HD	Stroke	HAD	ALD	
Cellular									
Macrophages and microglia	+	+	+	+	+	+	+	+	[2,16,52,60,62,71,116–118]
$CD4^+$ T cells	+		(+)					+	[22,23,118–120]
$CD8^+$ T cells	+/–		+	+				+	[25,60,118,121–123]
B cells	+							(+)	[118,124]
Neutrophils		+				+			[16,52]
Molecular									
7-Ketocholesterol	+								[31]
CD40	+		+	+					[125–127]
CD95	+/–					+			[73,128]
Complement	+	+	+/–			+			[16,56,129–131]
COX-2	+		+	+					[87,132,133]
Glutamate	+	+	+	(+)	+	+			[16,52,56,134–137]
Interleukin-1	+	+	+	+		+			[55,138]
TNF-α	+/–	+/–	+/–						[56,139–142]
TRAIL	+		+			+	+		[22,71,73,143]
Perforin	+						+		[123]
ROS, NO and $ONOO^-$	+	+	+	+	+	+			[16,52,76,77]

[a]Abbreviations: AD, Alzheimer's disease; ALD, adrenoleukodystrophy; COX, cyclooxygenase; HAD, HIV-associated dementia; HD, Huntington's disease; MS, multiple sclerosis; NO, nitric oxide; $ONOO^-$, peroxynitrite; PD, Parkinson's disease; ROS, reactive oxygen species; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumour necrosis factor. '+' indicates that there is evidence for involvement of a factor as an effector. Where a '+' is shown in brackets, weak evidence hints to a possible involvement of the effector '+/–' indicates that there is evidence that a factor is involved as an effector and, in parallel, the same factor also has opposite functions. Where no symbol is shown, there currently does not seem to be evidence either for or against an involvement.

and the most important excitatory neurotransmitter, glutamate. The crucial role of IL-1 has been confirmed in models of ischemia, neurodegeneration, traumatic brain injury and also MS [55,65]. Concerning its deleterious effects in pathology, IL-1 might have indirect neurotoxic effects by binding to microglia, astrocytes and oligodendrocytes, and subsequently inducing the expression and release of several mediators, most of which are neurotoxic, including reactive oxygen species (see later in this section), other pro-inflammatory cytokines and chemokines. Moreover, the direct effects of IL-1 on neurons include the enhancement of seizure activity and of Ca^{2+} entry through the NMDA receptor ion channel, which contribute to increased susceptibility of neurons in inflammatory conditions. IL-1 receptor antagonist (IL-1RA) was recently tested in a placebo-controlled clinical stroke trial, and indeed improved outcome in patients with cortical infarcts [66]. Thus, IL-1 is an attractive therapeutic target, because it represents a common inflammatory damage pathway in primary neurodegenerative and neuroinflammatory disorders, and can be pharmacologically modulated *in vivo* using the IL-1RA.

TRAIL

In principle, members of the TNF family can exhibit both neurotoxic and neuroprotective effects in pathological conditions. For example, a neuroprotective function was recently suggested for TNF, because neuronal damage caused by focal cerebral ischemia or epileptic seizures is enhanced in the absence of the TNF receptors (Table 2). However, whereas TRAIL has immunoregulatory functions outside the brain, its effects within the CNS are purely neurotoxic, crucially contributing to brain injury in different pathological conditions. Death-mediating TRAIL receptors are found on potential brain targets such as neurons and oligodendrocytes [67], and soluble TRAIL mediates neuronal and oligodendroglial death in human brain slices [68]. Human T cells and macrophages upregulate TRAIL expression upon activation [69], and TRAIL induces death of transformed neural cells [70]. Besides the previously mentioned role of TRAIL in $CD4^+$-T-cell-mediated collateral death of neurons in EAE [22], a recent study showed the contribution of TRAIL expressed by HIV-infected macrophages in a humanized mouse model of HIV. Neutralizing TRAIL but not TNF-α or CD95 (Fas) ligand blocked neuronal apoptosis [71]. Indeed, independent histopathological studies revealed a close association of TRAIL-expressing macrophages and apoptotic cortical neurons in HIV encephalopathy [72]. TRAIL expression is also found in apoptotic brain areas in rodent ischemia models, and blockade of TRAIL activity protects against ischemic neurodegeneration [73]. In AD, TRAIL expression is confined to affected brain regions, particularly in the proximity of amyloid plaques. Furthermore, contribution of TRAIL to Aβ-mediated neurotoxicity was recently demonstrated *in vitro* [74]. Thus, a targeted strategy to modulate TRAIL receptor–TRAIL interactions within the inflamed brain – a strategy not yet available for human studies – might form the basis of an appealing approach to the prevention of neurodegeneration in different pathological scenarios.

Reactive oxygen species and excitotoxicity

It is generally accepted that reactive oxygen species, comprising free radicals such as superoxide and nitric oxide (NO), can be found in virtually all pathological conditions that involve activated and neurotoxic immune cells, particularly macrophages and microglia. For example, NO has been implicated in a wide range of neurological disorders, be they inflammatory or degenerative (reviewed in Ref. [75]). NO can cause a reversible block of axonal conduction – presumably occurring in the acute relapse phase in MS – and an irreversible degenerative injury, especially to electrically active axons [76]. Indeed, NO is an important factor contributing to ongoing neuronal and oligodendroglial death, because it inhibits mitochondrial respiration, leading to intracellular accumulation of Na^+ and Ca^{2+}. Widespread mitochondrial dysfunction is central to current concepts of primary neurodegenerative diseases [77] and was recently detected in the brains of MS patients [78]. In fact, altered mitochondrial functions are a key feature of excitotoxicity found not only in primary neurodegenerative but also in neuroinflammatory diseases. Overactivation of excitatory glutamate receptors results in increased intracellular Ca^{2+} levels, leading to cytochrome *c* release and apoptosis. Pharmacological modulation of the glutamate system has been identified as an important therapeutic target (Table 2).

Inflammatory neurodegeneration – the perspectives

Collateral neuronal damage is clearly inherent to primary neuroinflammatory diseases, and neuroinflammation is a likely consequence of primary neurodegeneration. As outlined here, recent research efforts have successfully identified the intriguing overlap between such clinically heterogeneous diseases as AD and MS. However, to understand the mechanisms that initiate inflammation upon neurodegeneration and vice versa, several important issues will need to be clarified. For targeted therapeutic interventions, it is the task of molecular imaging to elucidate the precise kinetics – that is, the temporal and spatial contribution of the different inflammatory cells to pathology in the disease course. It is crucial to elucidate, on the molecular level, how and why T cells and microglia are differentially involved in different disorders. Experimental approaches amalgamating immunological and neurobiological aspects of disease are needed to understand the transition phases of inflammatory neurodegeneration. These approaches should seek to identify the individual molecular steps leading to inflammation-mediated neuronal death. From the data discussed here, it appears that in various neurological diseases the initial triggers differ considerably, whereas the subsequent pathways that involve inflammatory processes and cause brain damage share crucial pathological mechanisms. Targeting these processes, which arise from the interface of immune response and neuronal homeostasis, might lead to a quantum leap in the therapy of inflammatory and neurodegenerative diseases, which in the latter case has until now been even less satisfactory than the treatment of MS.

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